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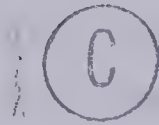
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MYOFIBRILLAR PROTEIN TURNOVER IN NORMAL AND DYSTROPHIC
MUSCLES IN CULTURE

BY



RICHARD ADAM JANECZKO

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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IN

ANIMAL BIOCHEMISTRY

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA

FALL, 1982



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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled MYOFIBRILLAR TURNOVER IN NORMAL AND DYSTROPHIC MUSCLES IN CULTURE submitted by RICHARD JANECKO in partial fulfilment of the requirement for the degree of DOCTOR OF PHILOSOPHY in ANIMAL BIOCHEMISTRY.

TO MY WIFE CAROL

ABSTRACT

The effects of the microbial protease inhibitors, leupeptin and pepstatin, on various aspects of protein metabolism in primary cultures of both normal and dystrophic chick embryo skeletal (leg) muscle, were investigated. Exposure of post-fusion, ^{35}S -methionine labelled cultures to either leupeptin or pepstatin for periods of up to 72 hours resulted in an inhibition of intracellular protein degradation as indicated by a reduction in the rate of loss of isotope for the total cell protein fraction. A return towards control levels of intracellular protein degradation during the latter stages of the experimental period indicated a decrease in the effectiveness of the inhibitors with time. This decrease was more apparent in cultures of dystrophic muscle. In addition, prolonged exposure (48 hours) of dystrophic cultures to a combination of leupeptin and pepstatin resulted in cytotoxicity and degeneration of the cells.

In order to examine more specifically the effects of the inhibitors on muscle contractile proteins a procedure for the isolation of myofibrils from the cultures was devised. Briefly, a suitable preparation of single sarcomeres and short myofibrillar fragments could be obtained by relaxation and induction of a rigor-like state prior to detachment and homogenization. Analysis of such preparations by SDS-PAGE indicated the presence of most major myofibrillar proteins, although the amounts of each increased disproportionately during early development.

Estimates of half-lives of four myofibrillar components from

normal cultures indicated non-uniform turnover of the myofibril. Similar results were obtained from cultures of muscle from dystrophic embryos. Treatment with either leupeptin or pepstatin resulted in an inhibition of the turnover of these components in both normal and dystrophic cultures. Again it was noted that the effectiveness of the inhibitors decreased with time during the experimental period and that this effect was more obvious in dystrophic cultures.

On the basis of these studies it is suggested that the microbial protease inhibitors, perhaps through action on proteases of the lysosomal system or in the case of leupeptin through inhibition of the Ca^{++} -activated neutral protease, act initially to block intracellular protein degradation. The cells subsequently respond, by mechanisms presently unknown, to overcome this inhibition and restore control levels of intracellular protein degradation, more so in dystrophic than in normal muscle. It is not known whether the difference in the capacity to restore control levels of intracellular protein degradation between normal and dystrophic cultures is attributable to the dystrophic gene.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
CAF	Ca ⁺⁺ activated factor
CANP	Ca ⁺⁺ activated neutral protease
CPM	counts per minute
DFP	di-isopropyl phosphorofluoridate
DMSO	di-methyl sulphoxide
DTT	di-thiothreitol
ECP	extractable cell protein
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene-bis-glycol-tetraacetic acid
HBSS	Hank's balanced salt solution
LC _{1,2,3}	myosin light chains (1,2 or 3)
MHC	myosin heavy chain
NCS	tissue solubilizer
NEM	N-ethylmaleimide
PCMP	p-chloromercuribenzoate
RIB	rigor inducing buffer
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNU	protein extraction buffer

T ₃	L-3,5,3' triiodothyronine
T ₄	L-3,5,3',5' tetraiodothyronine
TAT	tyrosine amino transferase
TCA	trichloroacetic acid
TM	tropomyosin
TN-T,C,I	troponin-T,C, or I
TSP	α -toluenesulphonyl phosphate

REVIEW OF LITERATURE

I. REVIEW OF LITERATURE

A. General Introduction

As with other tissues in the body, observations have led to the concept that protein turnover in muscle occurs as an integral part of normal metabolism, and that muscle cells possess, as a characteristic property, a mechanism not only for synthesis but also degradation of constituent proteins. The nature of the degradation mechanism is at present unknown, but it is believed to be the result of a process requiring the concerted action of intracellular proteases. Although studies have revealed the presence of a number of proteases in muscle, as well as in other tissues, it is not as yet been possible to link conclusively the activity of any one of these enzymes to the turnover process. The possibility that degradation may involve non-enzymatic reactions cannot be ruled out.

Much of the earlier work on protein turnover was concerned with the role of degradation in regulation of soluble cytoplasmic enzyme concentrations, particularly hepatic enzymes. This bias reflected not only an interest in possible modes of metabolic control through regulation of enzyme degradation rates but also, proteolysis of soluble cytoplasmic enzymes probably represents one of the simplest protein systems undergoing catabolism in a cell. Additional problems arise in the study and interpretation of the turnover of structural proteins. In such cases it is also necessary to take into account how degradation occurs during turnover, without disruption of the integrity and functionality of the structure from which the protein is derived. This problem is especially relevant to skeletal muscle,

where much of the intracellular protein is incorporated into the highly organized contractile apparatus, the myofibril.

Under steady state conditions the rates of synthesis and degradation are equal and no net change occurs in the level of protein within the cell. On the other hand in muscular dystrophy the tissue exhibits a net negative nitrogen balance which results in a progressive loss of protein and wasting of the muscle. In this case, and in a number of other experimentally induced muscular atrophies, the net loss of protein has been correlated with increased catabolic rates and elevated levels of specific proteases, but it is not known whether this represents the result of a relative increase in the activity of the normal catabolic processes occurring during turnover or whether mechanisms distinct to the pathological process are responsible. An understanding of the underlying mechanisms involved in these conditions is essential to a resolution of this problem.

B. Muscle Proteins

The soluble or sarcoplasmic fraction of skeletal muscle constitutes about 20%-25% of the tissue protein (Young, 1970). However, the bulk of intracellular muscle protein (~60%-65%) is accounted for by the myofibrillar fraction (Young, 1970; Goll et al., 1970). The nature of these proteins and the manner in which they are incorporated within the structure of the myofibril is known in some detail (Young, 1970; Goll et al., 1970). Each myofibril consists of a linear sequence of identical functional contractile units known as sarcomeres (Figure 1). The saromere in turn is a construction of thick and thin filaments



Figure 1. Diagrammatic representation of an
isolated myofibril and structure
of a single sacromere (Young, 1970).

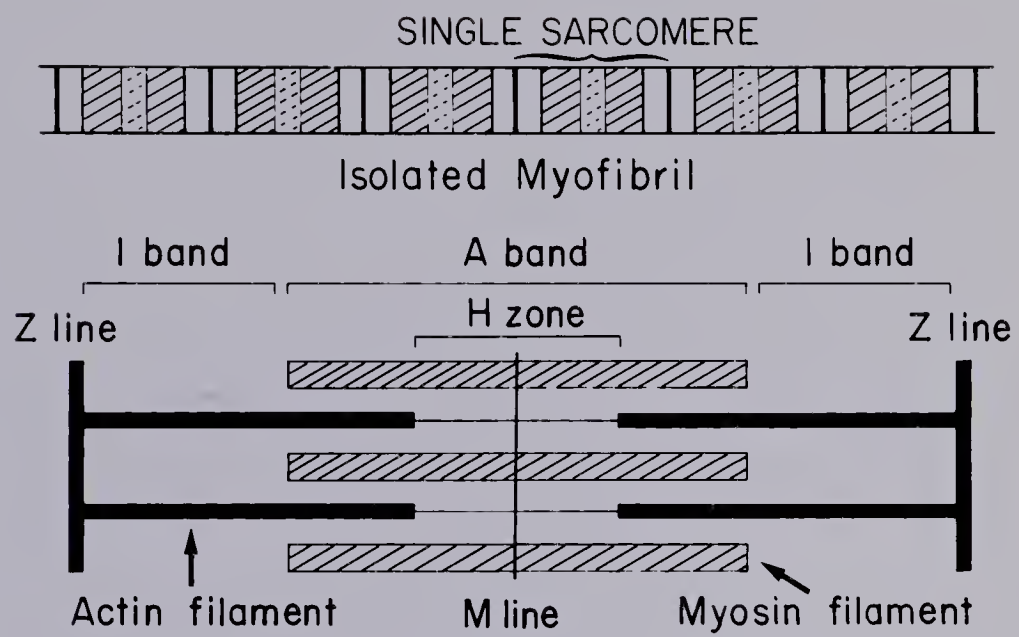


TABLE 1
THE MYOFIBRILLAR PROTEINS

PROTEIN	MOLECULAR WEIGHT	LOCATION
Myosin (Heavy chain)	200,000	Thick Filament
Myosin (Light chain 1)	*27,000/25,000	Thick Filament
Myosin (Light chain 2)	20,000/18,000	Thick Filament
Myosin (Light chain 3)	16,000	Thick Filament
C-protein	140,000	Thick Filament
Actin	45,000	Thin Filament
Tropomyosin (dimer)	70,000	Thin Filament
Troponin-T	37,000	Thin Filament
Troponin-I	24,000	Thin Filament
Troponin-C	20,000	Thin Filament
α -Actinin	100,000	Z-Line
Desmin	65,000	Z-Line
M Protein (M1)	190,000	M-Line
M Protein (M2)	180,000	M-Line
Creatine Phosphokinase	40,000	M-Line
Titin	1,000,000	uncertain

Data from Etlinger et al., 1976

* Light chains of slow/fast muscle

transected by the M and Z lines respectively. The areas of overlap between the filaments give rise to a dark anisotropic area referred to as the A-band whilst the region of thin filaments which is not overlapping is isotropic and is known as the I-band. The boundaries of the H-zone are variable and depend on the extent of penetration of the thin filaments into the A-band. During contraction and relaxation the sets of filaments slide past each other either causing shortening or lengthening of the sarcomere. The major proteins which are thought to be components of the myofibril are listed in Table 1.

Myosin is found in a polymerized form as the major constituent of the thick filaments. The properties of myosin have been shown to be variable and depend on a number of factors including the type of muscle from which it is derived and the stage of ontogenic development (Syrový, 1979). In general it has a molecular weight of about 500,000 daltons, is composed of a number of polypeptide subunits and possesses an ATPase activity and specific actin binding site (Syrový, 1979). The molecule is assymetric consisting of an α -helical coiled tail and a globular head in which are located the ATPase and actin binding sites.

Myosin derived from vertebrate fast muscle i.e. muscle with a rapid contraction/relaxation cycle (Locker and Hagyard, 1967; Sarkar et al., 1971) has a high specific ATPase activity and exhibits four distinct subunits on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The subunits correspond to a myosin heavy chain (MHC_f) and three light chains (LC_f) with approximate molecular weights of 200,000 daltons (MHC_f), 25,000 daltons (LC_{1f}), 18,000 daltons (LC_{2f}), and 16,000 daltons (LC_{3f}). The subunits MHC_f ,

LC_{1f}, LC_{1f}, LC_{2f}, and LC_{3f} are present in a ratio of 2 : 1 : 2 : 1. On the other hand myosin from slow muscle i.e. muscle with a slow contraction/relaxation cycle, has a relatively low specific ATPase activity and exhibits only three subunits on SDS-PAGE, all of which are distinct from those in fast muscle. In this case the subunits correspond to a heavy chain and two light chains with approximate molecular weights of 200,000 daltons (MHC_s), 27,000 daltons (LC_{1s}) and 20,000 (LC_{2s}). The stoichiometric ratio of MHC_s : LC_{1s} : LC_{2s} is 2 : 2 : 2.

In cross-reinnervating experiments in which fast muscles were reinnervated with motor neurones from slow muscles and vice versa, the enzymatic activities and subunit compositions of myosin changed to those of the muscle from which the nerve had originally been derived (Sreter et al., 1974; Weeds et al., 1974). In addition long term stimulation of fast twitch muscle at frequencies similar to those for slow muscle resulted in the synthesis of slow myosin (Sreter et al., 1975a). These experiments clearly indicated that the type of myosin synthesized by a muscle fiber was subject to neural regulation, but they did not distinguish between effects mediated through the pattern of impulses relayed by the nerve or to other factors such as diffusion of specific trophic substances. In an attempt to distinguish between these two possibilities John and Jones (1974) co-cultured rat embryonic skeletal muscle with spinal cord cells and examined the light chain composition of myosin. No difference could be detected between treatments and controls grown in the absence of the neural cells, suggesting that diffusion of trophic substances

was important under these conditions.

Initial investigations (Trayer and Perry, 1956) of myosin during ontogenic development concluded on the basis of ATPase activities and light chain composition, that embryonic myosin was more similar to adult slow than fast myosin. These findings were disputed (Dow and Stracher, 1971a, 1971b) on the grounds that the embryonic myosin preparations were not pure and that muscles with mixed instead of homogeneous fiber types (with respect to speed of contraction) were used. When the developmental changes were followed in a homogeneous muscle it was found that the embryonic slow muscle synthesized myosins similar to both adult fast and slow types (Pelloni-Mueller et al., 1976a, 1976b; Syrový and Guttman, 1977). However Dabrowska et al., (1977) have shown that the light chain analysis in these cases have been obscured by histone contamination of the embryonic myosins and that LC_{3f} was in fact absent in their earliest preparations. By contrast embryonic fast muscle synthesized only one type of myosin which was similar, but not identical to adult fast myosin. Its light chain composition differed only in that LC_{3f} was present in lower concentrations (Sreter et al., 1975b; Takahashi and Tonomura, 1975; Dabrowska et al., 1977). Comparative studies of MHC in embryonic and adult muscles have also demonstrated differences and it is possible that the embryonic form(s) of myosin are distinct from either adult fast or slow myosin (Gauthier and Lowey, 1977; Rushbrook and Stracher, 1979).

In all of these studies the tissue used was at a relatively late stage of development. Attempts to study earlier stages are limited

by technical difficulties and the fact that muscle development in vivo is a relatively asynchronous process, with a range of differentiating states existing in a muscle at any given time. This latter difficulty can be overcome to some extent by the use of muscle tissue culture. Using this system Rubinstein and Holtzer (1979) reported that cultures prepared from either precursor fast or slow muscle synthesized only one type myosin, which was identical with adult fast myosin in terms of immunological properties and light chain composition. On the basis of these results it was suggested that all pre-innervated muscles synthesize only fast myosin independent of their final developmental destiny.

Attempts to explain the changes occurring during development have led to the proposal that myosin exists in several isozymic forms which are coded for by different sets of genes (Holt and Lowey, 1977). Thus repression or stimulation of the specific sets of genes in response to various stimuli during development e.g. motor neuron maturation, would result in synthesis of different isozymes at particular developmental stages within a given cell. Recently, the successful fractionation of the postulated isozymes has provided evidence in support of this concept (Yagi et al., 1977). An interesting extension of the isozyme hypothesis has been proposed by Stracher, et al. (1979a). These workers and others have shown that myosin from dystrophic muscle is similar to embryonic "fast" muscle in terms of its ATPase activity and relatively low levels of LC_{3f} , and it was suggested that the switch from the embryonic to adult forms occurring during normal development, was either incomplete or did not occur in dystrophic

muscle. This failure could be due to either a breakdown in the normal genetic programme of the muscle or to other factors such as abnormal development of innervation. Clearly further studies will be required to identify the cellular mechanisms by which the synthesis of myosin is regulated and to answer questions about the relationships of pathological states to these processes.

In addition to myosin, it is now fairly certain that the thick filaments contain at least one other protein. Offer (1973) first purified C-protein (140,000 daltons) from preparations of thick filaments and since then several groups have confirmed this result. It is believed that C-protein assists in maintaining the ordered parallel alignment of the thick filaments but at present little else is known about it.

The thin filament of myofibrils is a co-polymer of actin and the regulatory proteins tropomyosin and troponin (Ebashi et al., 1969). Actin is a globular protein consisting of a single polypeptide with a sequence (Elzinga et al., 1973) molecular weight of about 43,000 daltons, and unlike myosin, actin from a wide range of sources shows very little variation in its properties (Pollard and Weihing, 1974). During contraction actin interacts with myosin to cause a sliding of the thin filaments into the interstices between the thick filaments (Huxley and Hanson, 1954). Under specified conditions monomeric actin (g-actin) will polymerize in the presence of ATP to form filamentous actin (f-actin). The g to f transformation results in the hydrolysis of the ATP and the incorporation of one ADP molecule between consecutive actin molecules in the polymer (Squire, 1975).

The major portion of the thin filaments is formed from two f-actin polymers coiled around each other. This results in the formation of a groove within which is found tropomyosin (TM) (Ebashi et al., 1969). Tropomyosin has a molecular weight of about 70,000 daltons, and contains two subunits of approximately equal molecular weights, which form a coil like structure similar to the tail portion of myosin (Lehrer, 1975). It exists in several polymorphic forms depending on the particular combination of the two known forms of the subunits, designated α and β . The α and β subunits differ slightly in sequence and can be separated on SDS-PAGE after carboxymethylation (Lehrer, 1975). As with myosin there is a correlation between the muscle type or developmental stage and the predominant polymorphic forms synthesized (Roy et al., 1979). However conflicting results on this matter have appeared in the literature. Thus, according to Roy et al. (1979), all embryonic muscles appear to synthesize mainly the β -subunit or β_2 form of TM, which is partly replaced by α -subunits during development, such that adult slow muscle contains approximately equal amounts of α and β subunits and adult fast muscle is almost exclusively α . Allen et al., (1978) on the other hand investigated TM synthesis in primary cultures of chick embryo skeletal muscle, and found a rapid rise in the synthesis of TM coincident with cell fusion. Both α and β subunits could be detected in these cultures, as was found for rabbit and chick skeletal muscle during development (Amphlett et al., 1976; Roy et al., 1976). The reason for the difference between these results and the previously mentioned studies is unknown and further investigation will be required to clarify this

matter.

The third component of the thin filaments is troponin (TN), which is located at regular intervals along the filaments, bound to TM. It is composed of three distinct subunits with molecular weights of 37,000 daltons (troponin-T), 24,000 (troponin-I) and 20,000 daltons (troponin-C). Troponin-T (TN-T) binds the complete molecule to the thin filament through TM, troponin-I (TN-I) is the inhibitory subunit which blocks the interaction of actin and myosin required for muscle contraction, and troponin-C (TN-C) is the Ca^{++} binding subunit which overcomes the effect of TN-I when Ca^{++} is released from the sarcoplasmic reticulum during contraction (Ebashi, 1974). There is now good evidence that all three troponin subunits exist in polymorphic forms which appear to be characteristic of the muscle type from which they are derived (Cummins and Perry, 1978). Unlike the cases with myosin and tropomyosin where more than one polymorphic form has been shown to be located within a single cell, only one form of troponin could be located by use of monospecific antibodies against TN-I in any given muscle fiber and it was suggested that this antibody staining technique could be used as an unambiguous method of muscle fiber typing (Dhoot et al., 1978). In a reference to unpublished observations Dhoot et al., (1978), stated that embryonic muscle differed from adult in that two forms of TN-I could be located in a single fiber, suggesting that one form is deleted during development.

A number of researchers have investigated the regulatory proteins in dystrophic muscle and in general it is concluded that there were no significant differences (Samaha, 1972; John, 1976). However, a

recent study by Irish et al., (1977) reported that antibody against TN-C did not react with their filament preparations from dystrophic muscle but it did react with those from normal muscle. Since the presence of a TN-C protein in the dystrophic preparations was confirmed by electrophoresis analysis, it was concluded that the failure to react with antibody must have been due to a structural alteration in the TN-C, possibly attributable to the dystrophic gene.

Estimates have shown that the Z-line accounts for about 6% of the dry matter of the myofibril (Huxley and Hanson, 1957), and there is a considerable body of evidence implicating α -actinin (102,000 daltons), and probably desmin (50,000 daltons), as integral components of this structure (Masaki et al., 1967; Lazarides and Hubbard, 1976; Lane et al., 1977). The remainder of the Z-line material, accounting for about 80% of the structure, has not as yet been conclusively identified, but the extreme sensitivity of the Z-line to proteases strongly suggests that it is composed largely of protein (Harsanyi and Garamovlgyi, 1969). Using a combination of differential extraction procedures and ultrastructural analysis of purified myofibrils Etlinger and Fischman (1973) and Etlinger et al. (1976) found that Z-line dense material could be rapidly extracted with 0.15% deoxycholate, leaving behind a filamentous skeleton. The extract contained two components (Z1 and Z2) with molecular weights close to 90,000 daltons and which they considered to be α -actinins. This rather low molecular weight appeared to be a characteristic of their system of electrophoresis analysis. The time scale of extraction of Z1 and Z2 differed from that of the dense material, with

the two Z proteins being extracted more slowly. It therefore appeared that the rapid loss of dense material was due to extraction of other unidentified Z-line components. Earlier investigations (Walcott and Ridgeway, 1967; Rash, et al., 1968) of Z-line composition had indicated that non-protein substances may form part of the Z-line and Etlinger and Fischman (1973) suggested that a part of the rapidly extracted dense material may have been of this nature. Analysis of the fibrillar residues remaining after A band and Z-line dense material extraction revealed the presence of only thin filament proteins. It therefore seemed possible that thin filament proteins e.g. actin, tropomyosin, formed an integral part of the Z-line, but attempts to confirm this using specific antibodies against actin and tropomyosin were unsuccessful. In studies with insect flight muscle it was found that isolated Z-lines contained considerable quantities of both actin and tropomyosin, but it was not clear whether this was simply due to contamination by thin filaments (Saide and Ullrick, 1974; Bullard and Sainsbury, 1977). More recently, Gard and Lazarides (1979) used a fluorescent labelling technique as a probe to investigate the components of the Z-line. Under appropriate reducing conditions the Z-lines of purified myofibrils could be specifically labelled with the fluorescent probe dansylcadaverine, in the presence of guinea pig transglutaminase. Analysis of labelled myofibrils by SDS-PAGE revealed fluorescence in 14 different bands including α -actinin, desmin, actin, tropomyosin and an unidentified 145,000 dalton component. Extraction of labelled myofibrils with 0.6 M KI to solubilize the thin filaments, did not result in solubilization of

Z-lines or the labelled tropomyosin or actin, suggesting that the labelled proteins were distinct from their thin filament counterparts and possibly located in or near the Z-line. In addition several ultra-structural investigations of the Z-line have proposed models in which there is a continuation of the thin filament with a lattice structure within the Z-line (Gard and Lazarides, 1979).

The other major transverse band of the myofibril, the M-band, has received somewhat less attention. Knappeis and Carlsen (1968) have proposed a model in which each thick filament, at the level of the M-line, is connected by a bridge structure to six surrounding thick filaments, and that consecutive levels of bridging are connected by a series of M- filaments running parallel to the thick filaments. The presence of amorphous material within the M-band was also noted and it was suggested that at least three components corresponding to each morphological feature of the M-line, could therefore be solubilized. Accordingly Etlinger and Fischman (1973) and Etlinger et al., (1976) found that M-line extraction from purified myofibrils resulted in the solubilization of five components, in addition to myosin, and designated them M1 to M5. Components M4 and M5 ($\sim 140,000$ daltons), were considered to be C-protein, whilst M3 ($\sim 150,000$ daltons) appeared to be a proteolytic fragment, probably derived from the myosin heavy chain. Porzio and Pearson (1977) have also observed a similar band on gels of whole myofibrils. Since components M1 (190,000 daltons) and M2 (170,000 daltons) were not found in purified thick filament fragments it was suggested that they were most probably derived from the M-line itself. Masaki and Takaiti (1972) reported the presence

of a single component in this molecular weight range in their M-line extracts and showed that antibodies against it reacted with the M-line of purified myofibrils. In addition to these proteins, there have been reports by several groups, on the presence of a 40,000 dalton component associated with the M-line (Eaton and Pepe, 1972; Etlinger and Fischman, 1973; Eppenberger et al., 1975). Investigations revealed that this protein was identical to creatine kinase and it seemed that in addition to its enzymatic activities, creatine kinase may also serve as a structural component of the M-line (Turner et al., 1973; Walliman et al., 1977). On the other hand the possibility that it was simply a cytoplasmic contaminant which became tightly bound during isolation, as is the case for phosphorylase (Arakawa et al., 1970), cannot be ruled out.

Thus although some progress had been made in the analysis of the M-line, as with the Z-line the definitive structure and composition remains to be elucidated.

A consistent feature of SDS-PAGE analysis of purified myofibrils is the presence of some very high molecular weight material, accounting for approximately 10% of the total myofibrillar protein (Etlinger et al., 1976), and which barely penetrates the top of the gel. Until recently this material tended to be regarded by most workers as cross-linked aggregates which were not properly dissociated by SDS treatment. However, Wang et al., (1979) have investigated this fraction and have concluded that these proteins are in fact distinct myofibrillar components. Myofibrils from various vertebrate and invertebrate species were found by SDS-PAGE to contain three high

molecular weight components, consisting of a doublet at about 1×10^6 daltons which they named titin and a smaller unnamed component at 500,000 daltons. They were shown to be chemically and immunologically distinct from actin, myosin and a contractile protein, filamin, from smooth muscle. Immunofluorescent staining of chicken breast myofibrils indicated that titin is localized in the Z-line, M-line, the A-I junction, and throughout the A-band. Similar staining patterns were obtained for myofibrils from other sources. Wang et al., (1979) speculated on the basis of these results that these proteins were structural components of the myofibril although their precise function is at present unknown.

C. Intracellular Protein Degradation

1. General Aspects

The realization that the rate of protein degradation may be quantitatively as important as the rate of synthesis in determining the tissue levels of proteins, has recently led to increased interest in the catabolic aspects of protein turnover. A number of general properties of the process have gradually come to be recognized and although much of this work was done on liver, it appears that the results are applicable to most tissue, including muscle (Goldberg and St. John, 1976; Waterlow et al., 1978).

A striking feature of protein degradation is that within a given tissue, there is a marked heterogeneity in the turnover rates of individual proteins (Schimke, 1975; Goldberg and St. John, 1976). This observation suggested that it is the intrinsic properties of the proteins which determine the rates at which they are degraded. How-

ever, Waterlow et al., (1978) have pointed out that similar and even identical proteins in different tissues exhibit different half-lives and that the relative "proteolytic environment" of the tissue from which the proteins are derived, is also important. In their terminology, the coarse control of degradation is effected through the proteolytic activity of the tissue, while the fine control is a function of the properties of the individual proteins and their susceptibility to the degrading system(s). Thus, in liver the coarse control setting is high and average half-lives short, while muscle has generally low levels of proteolytic activity and longer half-lives for individual proteins (Waterlow et al., 1978).

Under conditions where the degradative capacity of a tissue remains constant, changes in the protein complement could be brought about by alterations in the properties of specific proteins which in turn could result in their susceptibility to degradation. In this context, several properties of proteins have been identified which may act as determinants of their degradation.

Reports from a number of laboratories have shown that large proteins tend to be degraded more rapidly in vivo than small proteins (Dehlinger and Schimke, 1970, 1971; Goldberg and Dice, 1974). In addition, in multi-subunits proteins such as myosin (Zak et al., 1977) and fatty acid synthetase (Tweto et al., 1972), the larger subunits have been shown to turnover more rapidly. This finding led to the proposal that multi-component protein structures, which are in general not degraded as units, undergo a continual association/dissociation process and that degradation occurs in the dissociated state (Schimke, 1975) (see section on myofibril turnover). The fact

that larger proteins are degraded more rapidly may simply be a reflection of the fact that such molecules would be expected to have an increased probability of random encounter with a protease. This proposal has been supported by the observation that large proteins, and in general those proteins with shorter half-lives in vivo, are preferentially attacked in vitro by proteases such as trypsin and pronase (Schimke and Bradley, 1976). However, it appears that the conformational state of the protein is more important since the correlation does not hold when the proteins are denatured by urea and thiol reagents prior to digestion (Dice et al., 1973). Other factors which influence the conformational state of a protein have also been found to influence the in vivo turnover rates. Abnormal bacterial proteins resulting from structural mutations in lac repressor (Platt et al., 1970) and α -galactosidase (Goldschmidt, 1970) were shown to be rapidly and selectively degraded in vivo, and proteins which incorporate amino acid analogues during synthesis are degraded more rapidly in animal cells (Schimke and Bradley, 1976; Knowles and Ballard, 1976). Thus, rabbit reticulocytes incubated in the presence of 2-amino-3-chlorobutyric acid, an analogue of valine, synthesize an abnormal hemoglobin with a half-life of 15 minutes (Etlinger and Goldberg, 1977; Goldberg et al., 1978). In contrast normal hemoglobin is barely degraded within these cells. As mentioned previously, there is some evidence to suggest that proteins in genetically dystrophic muscle differ from those in normal muscle and it is possible that these abnormal proteins are relatively unstable due to conformational differences resulting from defective synthesis.

Similarly, there are numerous examples of the interaction of proteins with ligands and other small molecules, affecting their in vivo and in vitro stability. Schimke et al., (1965) showed that the interaction of tryptophan oxgenase with its substrate stabilized it against degradation in vivo and Litwack and Rosenfeld (1973) demonstrated a correlation between the in vivo turnover of several pyridoxal dependent enzymes and their affinity for pyridoxal phosphate. Similarly, Katanuma and his colleagues (1975), found that the degradation of ornithine transaminase by group specific proteases only occurred in the apo form of this enzyme. Based on these observations it was suggested that transformation from the holo to apo form may be a prerequisite to, and possibly the rate limiting step in, their degradation. More generally, proteins in vivo may exist in a number of distinct states, some of which are more susceptible to degradation than others, and the conversion from stable to susceptible forms could be necessary and rate limiting for proteolysis (Segal, 1975). Furthermore, the changing concentrations of substrates, co-factors and other ligands within a cell, in response to changing environmental conditions, would alter the equilibria of these interconversions, and this may regulate the turnover of specific proteins. Since these interconversions are viewed as being reversible, then they would be distinct from changes associated with molecular "aging" which would presumably mean irreversible changes in the protein. In addition, Schimke (1975) and Waterlow et al., (1978) have pointed out that the essentially first order turnover kinetics observed for most proteins are inconsistent with the idea of accumulation of damage or aging of proteins to explain why a given molecule is degraded.

An interesting and presently unexplained feature of intracellular protein degradation is its requirement for energy. The original studies of Simpson (1953) and Steinberg and Vaughan (1956) showed that inhibitors of energy metabolism such as cyanide and 2,4-dinitrophenol, also caused inhibition of protein breakdown in rat liver and kidney slices, and these results were subsequently confirmed by others (Hershko and Tompkins, 1971; Poole and Wibo, 1973). Similarly, protein breakdown in bacteria also appears to be an energy dependent process (Goldberg et al., 1973). Reports by Umana (1970) and Umana and Feldman (1971) of a direct effect of ATP on protein degradation as estimated by the release of ninhydrin positive material, in neutral rat tissue homogenates, were later shown by Goldspink and Goldberg (1973) to be erroneous. The observed increase in ninhydrin positive material was in fact due to ammonia production brought about by deaminase activity and not protein degradation. In addition, the ATP induced stimulation of proteolysis by isolated rat liver lysosomes observed by Natori (1975) may have been due to lysosomal disruption by ATP at pH 4.5 and release of proteases as reported by Huisman et al., (1974a). On the other hand, Mego (1973) has presented evidence that ATP may be involved in the maintenance of an acid milieu in lysosomes and recently an ATPase associated with the lysosomal membrane and possibly involved in this process has been reported (Schneider, 1977). However, as already pointed out protein degradation in bacteria which do not contain lysosomes is also an energy dependent process suggesting that the energy requirement is not related to lysosomal function.

In a study of the degradation of steroid induced tyrosine amino

transferase in hepatoma cell cultures Hershko and Tomkins (1971) demonstrate clearly an inhibition of breakdown concomitant with inhibition of energy metabolism by NaF, DNP and NaN_3 . More recently, Etlinger and Goldberg (1977) have reported a cell free soluble, ATP-dependent proteolytic system responsible for the degradation of abnormal hemoglobin in reticulocytes; the system did not degrade normal hemoglobin, was stimulated by physiological concentrations of ATP and appeared to be derived from the reticulocytes and not contaminating white cells also present in the preparation. Furthermore, it was active at pH 7.8 and did not exhibit latency indicating that it was not lysosomal in origin. Hershko and co-workers (1978, 1979) have studied the role of ATP in this system and found that it is required at or near the initial cleavage of the proteins. They have reported the resolution of the system into three components all of which are required for proteolysis (Hershko et al., 1979). In addition to a heat stable polypeptide of molecular weight 9,000 daltons, a high molecular weight component ($\sim 450,000$ daltons) which is unstable at 42°C and is stabilized by ATP, and a third protein stable at 42°C have been separated. Although the interaction of these components and the role of ATP in this system are presently unknown, the establishment of a cell free proteolytic system with an energy requirement represents a significant advance in this field.

A requirement for continuing protein synthesis for the degradation of some proteins has also been reported, but this is not a consistent observation. In an early study, Steinberg and Vaughan (1956) showed that analogues of phenylalanine, which were thought to inhibit protein

synthesis also resulted in an inhibition of proteolysis as measured by release of phenylalanine, in rat liver slices. Attempts to reproduce these results were unsuccessful (Brostrom and Jeffay, 1970) and Poole and Wibo (1973) were unable to show an effect on protein degradation in cultured fibroblasts treated with inhibitors of protein synthesis.

However, studies of hepatic enzymes, in particular tyrosine aminotransferase (TAT) by Schimke (1967) have shown that the rapid decay of TAT back to normal levels after glucocorticoid induction in vitro, are blocked by inhibitors of protein synthesis, apparently as a result of inhibition of degradation. Kenny (1967) also found that in untreated rats degradation of the rapidly turning over TAT could be completely inhibited by puromycin and he suggested that degradation of TAT required the presence of a specific protein with a short half-life. Using hepatoma cells in culture Hershko and Tomkins (1971) found that only the enhanced proteolysis of TAT induced in these cells by nutritionally deficient medium was inhibited by puromycin and cycloheximide, and that normal proteolysis was not affected. It was suggested that a labile protein is required for the enhanced rates of protein degradation. Studies by Goldberg (1971a) in bacterial systems led to the suggestion that inhibition of protein synthesis caused an accumulation of charged t-RNA which in turn inhibited protein degradation.

Gunn (1978) investigated the effects of protein synthesis on protein degradation, using two temperature sensitive mutants of Chinese hamster ovary and lung cells whose rates of synthesis were

extensively inhibited at non-permissive temperatures (40°C). Under these conditions there were little or no effects on proteolysis. However, as with Hershko and Tomkins (1971), enhanced proteolysis in nutritionally inadequate conditions were reduced by cycloheximide. It was concluded that the regulation of proteolytic activity did not require synthesis of some regulatory proteins, but did not rule out the possibility that the enhanced proteolysis was subject to influence by charged t-RNA species, since the defect in synthesis in both mutants could have resulted in accumulation of these molecules at non-permissive temperatures.

2. Muscle Aspects

Investigators of protein degradation in muscle have addressed themselves to three basic questions. First of all, what are the molecular characteristics and mechanisms which underly protein degradation in muscle, secondly what are the factors which control the rate of muscle protein degradation and thirdly what role does degradation play in both normal and pathological states of muscle growth and atrophy?

a. The mechanisms of protein degradation in muscle.

i. The Substrate. In view of the predominance of the contractile proteins in muscle it is not surprising that most of the studies in this area have been directed at the mechanism of degradation of the myofibril, although the turnover of other non-myofibrillar proteins has also received some attention (Booth and Holloszy, 1977; Swick and Song, 1974; Terjung, 1979).

The study of turnover in a highly organized structure like the myofibril, poses additional problems not encountered in simpler systems such as soluble cytoplasmic enzymes. For example, are the

individual components of the myofibril turned over independently or do they turn over as a unit? If the complete organelle turns over as a unit, then the half-lives of the individual components would be expected to be the same. Similarly, if specific parts of the myofibril were degraded as units e.g., thin filaments, then the components of these particular structures within the myofibril should have identical half-lives. Alternatively, if turnover of the components occurs independently then the half-lives should be different for each protein. (It is conceivable that the individual components could turnover independently and still exhibit similar half-lives, although the likelihood of such a coincidence is remote). As mentioned in the preceeding section, studies of some multi-subunit enzymes revealed a heterogeneity of turnover rates among the individual subunits, which led Schimke (1975) to suggest that such systems exist in a state of continuous association/dissociation, and that degradation occurs in the dissociated state. A given subunit could then be incorporated into the associated state several times before it was finally selected for degradation. Furthermore, the properties of the individual dissociated components would, according to the general scheme of Waterlow et al., (1978), provide the fine control for the rate of degradation of each component. However, conversion to the dissociated state introduces additional steps which are potential sites for the rate controlling reactions in the degradation mechanism.

Two approaches have been used to estimate the relative rates of turnover of individual myofibrillar proteins in a number of in vivo and in vitro systems. The most common method has been to determine

the extent of incorporation of a particular labelled precursor (usually an amino acid) into the proteins at a suitable time after administration. The extent of incorporation should be a reflection of the relative rates of synthesis which in the steady state would be equal to the relative rates of degradation. However, as pointed out by Waterlow et al., (1978) this only holds true as long as the specific activity of the precursor is greater than that of the proteins. Once the specific activity of the fastest turning over protein peaks, then the differences in extent of labelling will decline, and the capacity for discrimination will diminish.

In an early study, Velick (1956) used this approach to compare the turnover rates of myosin, actin and tropomyosin, and found them to be 1 : 1.5 : 2.4 respectively. He also studied the turnover of the proteolytic fragments of myosin, heavy and light meromyosin, and from this was able to deduce that the myosin heavy chain was turning over about four times more rapidly than the light chains. These results were in agreement with Schapira et al., (1956) who carried out a similar study. In a number of more recent investigations, the same pattern of heterogeneity among myofibrillar components was observed. Low and Goldberg (1973), found from their incorporation studies in incubated rat diaphragms that tropomyosin was synthesized about twice as fast as both actin and myosin, with actin being slightly slower than myosin. In this case, the light chains of myosin appeared to be turning over more rapidly than the heavy chain. Subsequently, Koizumi (1974), reported the in vivo turnover rates of seven myofibrillar proteins from rabbit muscles. Several different pre-

cursors were used in this study which showed the relative turnover rates of M-protein, troponin, tropomyosin, α -actinin, myosin, 10-S-actinin, and actin, to be 7 : 6.5 : 3.3 : 3 : 2.5 : 1.7 : 1 respectively, with their corresponding half-lives in days being 11, 12, 22, 24, 29, 44 and 75. Although the turnover of myosin subunits was not thoroughly examined in this study it was noted that in a single trial using C^{14} -glycine as a precursor, the turnover rates for all subunits were similar. The incorporation method has also been used to investigate turnover of myofibrils in rat cardiac muscle (Zak et al., 1977). In this case the order of labelling was myosin heavy chain = α -actinin = tropomyosin > actin > light chain 1 = light chain 2. This variation was ascribed not only to differences in rates of synthesis and turnover but also to the existence of a macromolecular precursor pool from which light chains were available for incorporation into complete myosin molecules. Thus, newly synthesized labelled light chains would be diluted within this pool prior to assembly into myosin and the myofibril, resulting in an apparently lower extent of incorporation during the early period after labelling.

The second method which has been used to study myofibrillar turnover is the double labelling technique of Arias, et al., (1969). In this procedure the proteins are initially labelled with a ^{14}C -amino acid and then some time later, just prior to sampling, a second tritiated amino acid is administered. The ratio of the specific activities of $^3H/^{14}C$ are then determined for each protein. Those with a high turnover rate would be expected to exhibit relatively high $^3H/^{14}C$ ratios since incorporation of tritiated amino acid and loss

of ^{14}C -amino acid would be greatest in these proteins. A problem with measurements of this kind is the reutilization of labelled precursor released on degradation, resulting in higher specific activities over extended periods of time. Recently, Zak et al., (1977) have critically evaluated this method for studies of myofibrillar turnover and compared it to the incorporation method. Several factors were shown to be important including the length of the labelling periods and the time between them. In addition, the sensitivity of the method was greatest for proteins with half-lives between 1.5 and 3.0 days, although by manipulation of the injection schedule it could be made effective for proteins with longer half-lives. When applied to cardiac myofibrils it was originally found that the myofibrillar proteins exhibited identical $^3\text{H}/^{14}\text{C}$ ratios (Zak et al., 1977). However, after re-evaluation of the method and comparison with incorporation measurements they found the turnover rates of several muscle proteins to be related as follows, myosin heavy chain = α -actinin = tropomyosin > actin > light chain 1 = light chain 2. These results were similar to those reported earlier by Wikman-Coffelt et al., (1973) for myosin and its subunits. In a related study Martin et al., (1977) estimated the half-life of cardiac myosin heavy chain from incorporation and decay measurements with labelled leucine. The values obtained from incorporation or decay measurements were similar being about 5.5 days. However, when the specific activity of the precursor pool was taken into account the half-life decreased to 2.7 days indicating that persistence of radioactivity in the precursor pool (perhaps due to reutilization of

label), was a problem in these measurements. Funabiki and Cassens (1973) have used an unusual modification of the double label method in which they administered both isotopes and subsequently discontinued one. Using this approach they found asynchronous turnover of the thin filament proteins with troponin > tropomyosin > actin.

Recently, turnover of myofibrillar proteins in primary cultures of muscle has been studied. Rubinstein et al., (1976) used the double label technique and concluded that actin and myosin heavy chain turned over at equal rates with half-lives of about 5-6 days. The method of isolation and analysis of the proteins studied was such that contractile proteins, not necessarily part of the myofibrillar apparatus of the cultured cells, would have contaminated the preparation and possibly distorted the results. Devlin and Emerson (1978), studied the activation of contractile protein synthesis during differentiation of quail muscle in culture, and found that several myofibrillar proteins including myosin heavy chain, myosin light chains, two troponin subunits and tropomyosin exhibited virtually identical molar rates of synthesis, while actin was found to be synthesized about three times more rapidly, in the steady state period, following cell fusion. Since those proteins which were synthesized at equal rates are not present in the myofibril in equimolar concentrations, it was suggested that there is either differential turnover or free pools of unassembled proteins that are present in the cells. Presumably differential turnover would be effected through different rates of degradation, but since the equal rates of synthesis were also observed during the steady state when rates of degradation would match

those of synthesis, then this cannot account for the differences in the pool size of the individual proteins within the myofibril. Thus, it seems likely that macromolecular pools of unassembled precursors, as already suggested by Zak et al., (1976) for myosin light chain, exist within muscle. If this is so, then the functional contractile apparatus represents only a fraction of the complete myofibrillar metabolic pool. Consequently, analysis of only those proteins within the actual myofibril may not be representative of the behavior of the total pool and erroneous results may be obtained. Interestingly, the procedures used by Devlin and Emmerson (1978) for analysis of the cultures were designed to extract the total cell protein and would therefore have included both assembled and unassembled precursor material.

On the whole measurements of the relative turnover rates of myofibrillar proteins have demonstrated heterogeneity among the individual components, suggesting that they are synthesized and degraded independently. In addition, there is some indirect evidence for the existence of a pool of unassembled macromolecular precursors (Zak et al., 1976). The observation by Etlinger et al., (1975) of two distinct populations of myofilaments, one of which is loosely associated with the myofibril and is apparently newly synthesized, may be related to this phenomenon. A factor which may be of importance in myofibrillar turnover but has as yet received very little attention, is the specific type of muscle involved. From what little evidence that is available (Waterlow et al., 1976), it appears that there is an inverse relationship between the rate of degradation and

the speed of contraction. However, the influence of such factors on the individual myofibrillar proteins, and their possible significance in turnover mechanisms are at present unknown.

A third method which has been used to investigate myofibrillar turnover, has been to pulse label the proteins and follow the loss of label occurring during turnover. This approach generates decay curves from which it is possible to calculate turnover rates and to obtain information about the type of kinetics involved in turnover, i.e. the manner in which label is lost from the proteins as a function of time. Two types of decay can be constructed, by following either the loss of activity due to degradation, or the loss of specific activity due to synthesis. Both of these methods are subject to error due to reutilization of label resulting in lower decay rates and apparently longer half-lives.

In terms of kinetics two broad possibilities can be envisaged. If the myofibril has a definite life-span then the total amount of label in the pool should remain constant for a period equal to the life-span following which it would be rapidly lost. Such a mechanism implies that the myofibril undergoes a process of aging, which could result from specific chemical or conformational modifications to the proteins increasing their susceptibility to degradation. Aging could also be viewed in terms of the specific location of components within the myofibrillar structure or the position of the whole myofibril within the cell. That is to say there may be an irreversible translocation of myofibrillar proteins through the contractile apparatus to specific sites of degradation within the

cell. On the other hand, it is possible that the individual components of the myofibril turn over randomly and do not exhibit aging, as has been found for a number of enzymes in other tissues (Schimke, 1975). Such a model would be compatible with the association/dissociation mechanism described earlier.

To date the only study indicating life-time kinetics for myofibrils was that reported by Dreyfus, et al., (1960). After a single injection of either labelled glycine or valine into rats the activity in myosin remained constant for 30 days after which it declined to a new level. Although it was shown that reutilization of label was occurring in this experiment it is not clear whether this was sufficient to account for the sustained presence of label over the 30 day period. In a similar study, Kimata and Morkin (1971), found little or no loss of label from muscle myosin in adult rats over a period of 45 days. After correction for recycling of label they calculated a half-life of around 165 days. With such low turnover rates it would be difficult to observe significant loss of isotope from myosin pool, giving the impression of life-time kinetics.

Most other studies in this area (Wikman-Coffelt, 1973; Swick and Song, 1974) have found exponential decay of label indicating random turnover. In addition, the half-lives of myosin fell in the range of 5-165 days, which are considerably shorter than the estimates obtained from incorporation measurements (Zak et al., 1976, 1977; Martin et al., 1977). Interestingly when the decay method was used to study myosin turnover in primary cultures of chick skeletal muscle, half-lives ranging between 36 hours and 7 days were observed depend-

ing on culture conditions (Walker and Strohman, 1978). In addition, exponential decay kinetics were also observed. A possible explanation for the shorter half-lives in cultures could be that recycling of label was reduced by swamping the system with cold leucine, the precursor used in the study.

In summary then, most studies of myofibrillar turnover kinetics have indicated that turnover is random and that most likely aging does not play a role in turnover of the myofibril. These observations taken together with the variability of turnover rates would tend to exclude the possibility of turnover as a unit. Instead the picture which seems to be emerging is that the myofibril and the specific structures within it are continuously assembled and disassembled from precursor pools of the individual contractile proteins and that random selection for degradation probably occurs from the disassembled protein pools. Dayton et al., (1975) and his colleagues have proposed that the disassembly process may be controlled through the activity of a Ca^{++} activated protease, which acts in a "nicking" fashion to loosen the intact myofibril, and make available released components for mixing with the macromolecular pools and possibly proteolysis. However since there is no evidence at present to suggest that peptide or other covalent bonds between the individual contractile proteins, are involved in maintaining the integrity of the myofibril then the action of a protease may not be necessary for disassembly. In this context the observations of Hikida (1979), that Z-line stability is critically dependent on Mg^{++} concentrations rather than Ca^{++} , which was thought to destabilize the Z-line through activation of the

protease, are of interest. Similarly the studies of Etlinger and Fischman (1973) on isolated, protease free myofibrils have shown that the structure can be effectively disassembled using procedures which disrupt non-covalent intermolecular forces.

Based on this model of the total myofibrillar metabolic pool consisting of the intact organelle, the disassembled macromolecular components and the free amino acids, two general possibilities for rate controlling sites can be proposed. This could occur at the level of either assembly/disassembly or through degradation of the free macromolecular constituents. Certainly in the latter case proteases, whose activity could be important in rate control, are involved. On the other hand the mechanisms and enzymology of the proposed macromolecular disassembly remain to be elucidated. In any event it seems important that future investigations of myofibril turnover should take these concepts into account.

ii. The Enzymology (Proteases in Muscle). The intracellular degradation of muscle proteins occurring during normal turnover is believed to be brought about by the action of intracellular proteases. Although a number of such enzymes have been identified in muscle tissue their possible involvement in protein turnover remains to be elucidated. Compared to other tissues such as the liver the level of proteolytic activities in muscle is relatively low. For the purposes of discussion only, these activities can be divided into three groups, the acidic, neutral and alkaline, according to their in vitro pH optima. Recent reviews in this area have been published by Bird (1975, 1980) and Pennington (1977). Examples of acid, neutral and

alkaline proteases found in various tissues are given in Table 2.

The better characterized proteases are now usually classified according to important features of the active site (Table 2 and see Barrett, 1977).

a. The acidic proteolytic activities. The acidic proteolytic activities of muscle, which are optimal below about pH 5.5, are most probably lysosomal in origin and would be expected to include proteases of the cathepsin series, the two best known forms being cathepsins B and D (Barrett, 1978, 1980), but cathepsins H and L may also be important (Bird et al. (1980). Recent studies by Bird et al., (1978) have demonstrated, by a combination of cytochemical and electron microscope techniques, specific sites of cathepsin B and D activity in muscle, which were coincident with lysosome-like structures. Two types of lysosomal structures were identified, one which appeared continuous with the sarcoplasmic reticulum and was embedded between myofibrils, and the other which was perinuclear in the vicinity of the Golgi apparatus. This close association of the lysosomal system and sarcoplasmic reticulum has been observed by others (Topping and Travis, 1974; Hoffstein et al., 1975) and appears to be a morphological adaptation specific to muscle. In addition, the proximity of some of these structures to the Golgi apparatus suggests this as a likely site of lysosomal synthesis, as has been implied for other tissues (Cook, 1973; Smith and Van Frank, 1975). Canonico and Bird (1970) have also isolated, by rate zonal centrifugation a population of lysosomes which they argued were derived from muscle fibers, and were shown to contain a fairly typical complement of lysosomal

enzymes, including cathepsins B and D. These results are in contrast to those from earlier investigations, which failed to obtain evidence for a lysosomal system in muscle, and they refute the suggestion that the low levels of lysosomal enzymatic activities obtained from muscle preparation, were in fact derived from non-muscle cells also present in the tissue (Pelligrino and Franzini, 1963).

Several studies have addressed themselves to the question of the proteolytic capacity of lysosomes and lysosomal enzymes. Bohley et al., (1974) incubated intact rat liver lysosomes at pH 6.9 with a high molecular weight liver cytosol protein fraction and observed degradation equivalent to about 2% protein/hour, a rate in excess of the normal in vivo rate. The cytosol protein fraction was used as substrate because it was considered more suitable than the more commonly used purified protease substrates such as serum albumin or hemoglobin. Dean (1975a) also used a similar cytosol fraction as substrate for purified rat liver cathepsins B and D and a soluble mixture of lysosomal enzymes. The results showed that the relative rates of in vitro degradation of cytosol proteins by the purified enzymes on the unfractionated mixture, correlated well with their in vivo rates of turnover. By contrast Huisman et al., (1974b) using a very similar protocol concluded from their studies that cathepsin D was not essential for the degradation of protein by liver lysosomal enzymes, but that cathepsin B and possibly other thiol proteases were important. In addition they concluded that liver lysosomes contain a set of

proteases sufficient for rapid and extensive degradation of albumin, the substrate used in these studies. The failure to obtain any effect with cathepsin D was probably due to the fact that cathepsin D shows very little activity against native albumin at pH 5.0, the pH of their assay medium. Initial attempts to demonstrate susceptibility of muscle to lysosomal proteases were unsuccessful (Sharpe, 1963; Park and Pennington, 1967; Friedman et al., 1969). However Schwartz and Bird (1977 and Bird et al., (1978) found that purified cathepsins B and D from either liver or muscle, were capable of degrading myofibrillar proteins as judged by SDS-PAGE. Interestingly the degradation of actin and myosin by cathepsin D was found to be more extensive than that by cathepsin B. They concluded from calculations based on these results that the levels of activity of these enzymes in muscle was sufficient to account for a significant amount of intracellular protein degradation in muscle.

A variety of techniques have been used to assess lysosomal involvement in intracellular protein catabolism in living muscle systems. By various perturbations the rates of proteolysis in the tissue have been varied and compared with changes in the lysosomal activity of the tissue. Under conditions of both physiological and pathological stress the presence of a lysosomal-like system becomes more readily apparent and can be demonstrated both biochemically and microscopically (Bird, 1975). Treatments such as starvation (Bird, 1975), denervation (Pellegrino and Franzini, 1963; Guttman, 1973), and tenotomy or immobilization

(Jirmanova and Zelena, 1970) result in large and often rapid alterations in the protein content of the tissues accompanied by characteristic increases in the size and number of lysosomal-like bodies and autophagic vesicles. Similar changes were found in genetically dystrophic muscle, although in these cases the time course of the changes was much protracted (Pearson and Kar, 1979). The net negative nitrogen balance of the muscle in both starvation and denervation appeared to have been partly due to increases in rates of protein degradation and may have been related to subsequently observed increases in the levels of cathepsin B and D (Bird, 1975). In dystrophic muscle the levels of these two enzymes as well as other lysosomal and non-lysosomal proteases were reported to be elevated (Pearson and Kar, 1979; Kar and Pearson, 1977). Evidence has also been presented for the involvement of lysosomes in conditions such as regression of thyroid induced cardiac hypertrophy (Wildenthal and Mueller, 1974) and post-partum involution of uterine smooth muscle (Lahav et al., 1977). In both of these cases the tissue exhibits an increased rate of proteolysis and a net negative nitrogen balance.

There is therefore considerable evidence to suggest that lysosomes may be involved in accelerated protein breakdown, in muscle subjected to relatively extreme environmental stress, but the mechanisms involved in these gross changes may not be the same as those associated with normal steady state conditions. Dice and Walker (1978) have shown that increased protein degradation in skeletal muscle during diabetes and starvation

may not be simply an acceleration of normal proteolytic mechanisms and Amenta et al., (1978), working with cultured fibroblasts has distinguished between the basal proteolytic mechanisms involved in normal turnover and those associated with gross changes induced by nutritionally deficient (serum depleted) medium. Results of studies in other systems have drawn similar conclusions (Warburton and Poole, 1977; Ward et al., 1977). Another problem with these studies is that they provide only indirect circumstantial evidence for the involvement of lysosomal enzymes in intracellular protein degradation independent of whether it is the basal type of degradation or otherwise.

A more direct approach to these problems has been suggested by the studies of Dean (1975b) on protein degradation in perfused livers and cultured macrophages. Using a specific low molecular weight protease inhibitor pepstatin entrapped in liposomes, Dean, was able to block protein degradation in perfused rat livers by up to about 50%. Pepstatin specifically inhibits carboxyl proteinases, of which cathepsin D is an example, and since cathepsin D is quantitatively the most important acid protease in liver it was assumed that blockage of protein degradation was due to inhibition of this enzyme. The fact that pepstatin was only effective when entrapped within liposomes, which are thought to enter cells either by fusion or pinocytosis and subsequently fuse with lysosomes, lent further support to the idea of a lysosomal site of action for pepstatin. Dean reported these results as the first direct evidence for lysosomal involvement in intracellular

protein catabolism. However there is now good evidence (Mortimore et al., 1978) to suggest that the perfused liver system used in this study was exhibiting accelerated proteolysis over and above that associated with normal turnover. More recently Dean (1979, 1980) has used a similar approach to evaluate the role of lysosomes in basal protein degradation in cultured mouse macrophages. Rates of proteolysis in these cells could be increased over basal levels by use of serum-depleted (step-down) medium. Step-down induced increases in proteolysis have been observed for other cell types in culture but the effect on macrophages is comparatively small being of the order of 5%-10% (Amenta et al., 1978). The presence of pepstatin in the complete medium resulted in a progressive inhibition of the rate of basal degradation reaching a maximum of about 40% after 48 hours. This was paralleled by a decline in the recoverable cathepsin D activity in cell suspensions. A second inhibitor leupeptin which has a broader specificity, and is active against cathepsin B, also induced inhibition of degradation but in this case the inhibition (25%-35%) reached a maximum within 4 hours. The temporal difference in the inhibitory effects of leupeptin and pepstatin was attributed to the fact that leupeptin which is water soluble probably permeated the cell membrane fairly rapidly while pepstatin which is virtually insoluble in water (1% methanol was used to solubilize it in the medium) appeared to enter the cell by a much slower pinocytic process. Several agents which affect the cytoskeletal structure of cells were also found to cause inhibition of degradation and it

was suggested that this may have been related to effects on non-enzymatic aspects of lysosomal autophagy in these cells. Dean concluded from these studies that lysosomes and the lysosomal proteases cathepsin D and B, were involved in basal protein turnover in cultured macrophages. The question as to whether the mechanism of degradation involved in the observed basal proteolysis is the same as that involved in normal turnover in vivo remains unanswered by these studies. Cells grown in culture are not subject to the very fine homeostatic controls of their in vivo environment and it is possible that even the basal proteolysis observed under nutritionally adequate conditions is simply a minimization of a generally accelerated proteolytic activity characteristic of cultured cells (Hendil, 1977). Nonetheless these studies demonstrated the potential of the protease inhibitors as tools for the evaluation of specific tissue protease involvement in intracellular protein degradation, and in recent years their effects on a number of muscle systems have been investigated.

Iodice (1976) found that pepstatin inhibited both preparations of cathepsin D from dystrophic chicken breast muscle and autolytic activity in homogenates of the same. He suggested that the inhibitor may have use as a therapeutic agent in retarding protein catabolism in dystrophic muscle. Subsequently McGowan et al., (1976) showed that pepstatin, leupeptin and antipain were capable of delaying degeneration and preserving the ultrastructure of normal and dystrophic explant cultures of chick embryo breast muscle. They also reported that similar but less striking effects

were observed in monolayer cultures of normal muscle, and that intracellular acidic protease activity (probably cathepsin D) and cathepsin B activity of the cultures could be inhibited by pepstatin and leupeptin (Stracher et al., 1979a). In addition they found that denervation atrophy in vivo could be delayed by administration of these inhibitors (Stracher, et al., 1979b). In a series of clinical studies in dystrophic chickens, Stracher et al., (1978) reported significant improvements in treated animals particularly with regard to the histological appearance of the tissue. However Enomoto et al., (1977) was unable to obtain evidence of improvements in dystrophic mice treated with leupeptin, pepstatin, and antipain, for periods greater than 300 days. Iontophoresis of pepstatin failed to prevent degeneration of insect intersegmental muscle which undergoes resorption during metamorphosis (Lockshin, 1975), although total acid protease activity in cell homogenates prepared from treated muscles was reduced by 54% with respect to controls. Other evidence (Lockshin and Beaulaton, 1974a, 1974b) has shown a proliferation of autophagic vesicles and increases in acid protease activity just prior to and during resorption suggesting a lysosomal involvement in this process. The failure to retard degeneration in spite of the demonstrated inhibition of acid protease activity may be explained by the inability of the aqueous insoluble pepstatin to carry current and consequently to enter the cells during iontophoresis. Thus it may simply have become adsorbed onto the cell and contact between enzyme and inhibitor only occurred during homogenization of

the tissue.

In an attempt to correlate actual rates of protein degradation in muscle with specific lysosomal proteases Libby and Goldberg (1978) and Libby et al., (1979) studied the effects of leupeptin and pepstatin on several normal and dystrophic in vitro muscle systems. Only leupeptin was found to be effective in retarding protein degradation as measured by the release of tyrosine, in any of the systems studied. Both normal skeletal and cardiac muscles from adult rats incubated in vitro showed inhibition up to 60% and 18% respectively. Protein synthesis was unaltered in these muscles. In addition cathepsin B activity was inhibited by about 35% suggesting this enzyme as a principle site of action of leupeptin. Similar results were obtained for denervated rat muscles and genetically dystrophic mouse muscles. Leupeptin also effectively inhibited (~50% inhibition) protein degradation in fetal mouse hearts during a period of 48 hours in culture. Interestingly, cathepsin B activities in this case were found to have increased significantly with respect to controls, at 24 hours and 48 hours in spite of the fact that proteolysis during this period continued to be inhibited. Inhibition effects on cathepsin B activity in the very early stages of culture corresponding to the incubation period studied in the adult tissue were not reported and it was suggested that the increase in cathepsin B in the fetal hearts was a relatively specific compensatory response to the sustained presence of leupeptin. Alternatively the proteolysis measured in this system may not have required cathepsin B and

leupeptin may have been active against a different enzyme. In a similar study Wildenthal et al., (1978) found that agents such as chloroquine and non-metabolizable sugars, which are known to cause lysosomal dysfunction also resulted in an inhibition of protein degradation in cultured fetal mouse hearts. In the particular case of chloroquine, observable alterations in mitochondria occurred which may have caused impairment of energy metabolism and thus through energy shortages inhibited protein degradation, an energy requiring process.

The results of these investigations suggested a lysosomal involvement, probably through cathepsin B, in intracellular proteolysis in these muscle systems but they do not exclude other non-lysosomal involvement. The rather broad specificity of leupeptin certainly allows for the possibility of extralysosomal sites of action. The failure to obtain inhibition with pepstatin may again be a reflection of the solubility of this agent and its general inability to enter cells. Even incubation periods of 48 hours, shown to be successful for macrophages did not produce inhibition with pepstatin, but this may be explained by the fact that the activity of the phagocytic mechanism by which pepstatin is probably taken up is in general low in muscle compared to tissues containing macrophage and reticuloendothelial cells. With respect to the proteolysis measured in these studies, it is likely that increased proteolysis over normal rates was occurring and this may have activated mechanisms not associated with normal turnover. In particular the fetal heart cultures were in extreme

negative nitrogen balance and atrophied quite markedly throughout the experimental period. Consequently conclusions about lysosomal involvement in normal muscle protein turnover cannot be answered by these investigations.

Aside from its rather obvious potential as an agent of intracellular protein catabolism, there are some general properties of the lysosomal system which are difficult to reconcile with known characteristics of turnover. It is unlikely that the acidic condition required by the lysosomal proteases for optimal activity occurs extralysosomally in muscle. Estimates of the in vivo intralysosomal pH have indicated values as low as pH 4.0 which would be sufficient for significant levels of protein degradation (Mego, 1973; Bird et al., 1978). It therefore seems that ingestion of substrate into the lysosome would be the necessary mode of action rather than leakage of proteases into the cytoplasm. A second problem is that under given physiological conditions the individual proteins of tissues exhibit distinct half-lives and it is difficult to see how the rather indiscriminate digestive activity of the lysosome could account for this. Several possible regulatory mechanisms have been proposed (Segal, 1975; Dean and Barrett, 1976; Kay, 1978) including selective uptake of substrate by the lysosome, but such suggestions have yet to be confirmed. An alternative explanation is that if lysosomes play a part in normal turnover then they may be involved in the terminal post-regulatory steps and selectivity controls determining individual half-lives occur extralysosomally prior to lysosomal ingestion. In this context the

neutral cytoplasmic proteolytic activity of muscle has been implicated as having an important function, particularly with respect to myofibrillar turnover.

b. The neutral proteolytic activities. A number of proteases optimally active at neutral pH have been isolated from muscle tissue (Huston and Krebs, 1968; Kohn, 1969; Busch et al., 1972). All of these enzymes required Ca^{++} for activation and there is reason to believe that they are probably the same or at least closely related enzymes. The preparation by Busch et al., (1972) known as Ca^{++} activated factor (CAF) has been most extensively investigated (Dayton et al., 1975, 1976a, 1976b). Using porcine skeletal muscle as a source they found it had a molecular weight of 112,000 daltons and is composed of equimolar amounts of 80,000 dalton and 30,000 dalton subunits. Preparations of CAF from other mammalian sources including human (Reddy et al., 1975; Suzuki et al., 1979) were similar but it was noted that a chicken muscle preparation (Ishiura et al., 1978) contained only the 80,000 dalton subunit. The pH optimum of the porcine enzyme against myofibrils or caesin substrates was pH 7.5, and required the presence of 1 mM Ca^{++} and 2mM β - mercaptoethanol. The enzyme was inhibited by iodoacetamide and leupeptin suggesting it is a thiol protease with a calcium ion requirement.

CAF was discovered through its ability to selectively attack intact myofibrils and specifically remove the Z-line (Busch et al., 1972). The characteristics of this specificity were investigated by incubation of purified CAF with myofibrils,

or purified myofibrillar protein, and subsequent analysis by SDS-PAGE. With whole myofibrils almost all of the α -actinin was lost within 5 minutes, and degradation of C-protein and troponin-I was almost complete. Simultaneous with these changes was the appearance of a band at 30,000 daltons. Smaller decreases in the amounts of tropomyosin and troponin-T was also observed. Incubating with purified myofibrillar proteins showed that CAF was unable to degrade myosin, actin or α -actinin. On the other hand degradation of C-protein, tropomyosin and the troponin-I and troponin-T subunits of troponin did occur. This limited proteolytic attack contrasts with the more extensive and indiscriminate digestions produced by proteases such as trypsin (Dayton et al., 1975).

The inability of CAF to digest α -actinin suggests that the loss of this protein from intact myofibrils is due to its solubilization rather than hydrolysis as has been shown by others (Reddy et al., 1975). In addition this probably accounts for much of the concurrent loss of Z-line integrity. The 30,000 fragment which appeared on digestion of whole myofibrils, was probably the same as that derived from troponin-T during incubation of purified TN with CAF. An interesting feature of CAF digestion of myofibrils is that its principle sites of action within the myofibril are all related to maintenance of the structural integrity of the organelle. This observation has led to the proposal that CAF acts to loosen the myofibrillar structure by attacking these strategic structural components and causing the release of fragments for subsequent

degradation by other systems such as the lysosome (Dayton et al., 1975).

Previous studies by Etlinger et al., (1975) had shown that treatment of isolated myofibrils with an ATP-relaxing solution resulted in the release of a small amount of thick and thin myofilaments probably from the periphery of the myofibril. In vitro incubation of skeletal muscle with the Ca^{++} ionophore A23187 resulted in increased proteolysis in the muscle and at the same time increased the size of the population of releasable myofilaments obtained from myofibrils prepared for these muscles (Kameyama and Etlinger, 1979). Incubation of purified myofibrils with CAF also increased the quantity of releasable myofilaments. In both of these cases the increase could be inhibited by leupeptin which also reduced proteolysis in the in vitro incubated muscles. Thus it is possible that the releasable myofilaments are intermediate in the disassembly of the myofibril and that their release is brought about by CAF, as suggested above.

There is at present very little information on the regulation and control of the enzyme in response to various physiological perturbations. Dayton et al., (1977) has shown that the activity of CAF is increased in muscles from rabbits with nutritional (vitamin E deficient) muscular dystrophy. In a study of a similar Ca^{++} activated protease in cardiac muscle Waxman (1978) reported that the activity was often masked by two polypeptide inhibitors which co-purified with the enzyme. One of the inhibitors (270,000 showed a high degree of specificity for the cardiac Ca^{++} activated

protease and it was suggested that such inhibitors may serve as an in vivo control mechanism, not only for this enzyme but also other non-lysosomal proteases.

Although the limited and specific mode of action of CAF on myofibrils makes it an attractive candidate for the task of initiating the disassembly of the myofibril there are certain features of the enzyme which raise doubts as to its physiological significance. First of all the Ca^{++} requirement of 1.0 mM is at least an order of magnitude greater than that found in muscle. In accordance with the idea that intracellular Ca^{++} levels are elevated due to a membrane defect in hereditary muscular dystrophy, (Cullen et al., 1979; Wroegman et al., 1979) it was proposed that these conditions would lead to stimulation of CAF and perhaps account for part of the accelerated protein degradation in dystrophic muscle. However even in dystrophy Ca^{++} levels would not rise above 0.1 mM, a concentration too low to elicit CAF activity. In addition to this difficulty the temperature profile of the enzyme is unusual in that it has a maximum at 10°C and shows almost no activity at 37°C. It appears that in the presence of Ca^{++} at 37°C the enzyme is unstable and degrades very rapidly (Goll, 1979, personal communication). Unless these objections can be overcome it is difficult to envisage any physiological role the enzyme might have to play in myofibrillar turnover and the search for suitable alternatives will continue.

c. The alkaline proteolytic activities. The third group of proteases, active in the alkaline range is somewhat more difficult to evaluate. A number of such activities have been reported but it is

not clear how many distinct enzymes are involved. All but one are serine proteases with pH optima in the range pH 8.5-10.5, they are insoluble in low ionic strength buffers and several are tightly bound to the myofibril. There are differences in molecular weights, effects of inhibitors, metal ion requirements and cell fractions from which they are isolated, indicating that more than one enzyme is involved.

Koszalka and Miller (1960a, 1960b) partially purified an autolytic activity with an optimum between pH 8.5-9.0 from the 10,000 x g. supernatant (post-myofibrillar) which had a chymotrypsin-like specificity. Subsequently Noguchi and Kandatsu (1970, 1971) reported an alkaline autolytic activity in rat skeletal muscle, which appeared to be associated with the myofibrils. The purified enzyme had a molecular weight of 22,000 daltons, pH optimum 9.0-10.5 and was also chymotrypsin-like in its specificity (Noguchi and Kandatsu, 1976). Holmes et al., (1971) reported an alkaline protease (25K) (pH 10.0) associated with rat skeletal muscle myofibrils, with properties similar to Noguchi and Kandatsu's enzyme. They did however differ in that the supernatant fraction strongly inhibited Noguchi and Kandatsu's enzyme (Noguchi and Kandatsu, 1970) but did not affect the preparation of Holmes et al., (1971). It was noted that these enzymes resembled in many respects a chymotrypsin-like protease found in peritoneal mast cells (Kawiak et al., 1971), and it was suggested that the muscle enzymes may in fact originate from mast cells present in the muscle. Thus attempts to isolate the enzyme from the muscle of rats previously treated with an agent

which selectively degranulates mast cells, were unsuccessful and it was concluded that the enzyme was probably derived from the mast cells (Park et al., 1973). Further support for this idea was provided by Drabikowski et al., (1977) who showed that the presence of the alkaline protease could only be demonstrated in muscles from animals known to have tissues infiltrated with mast cells. Accordingly, no activity is found in rabbit skeletal muscles, which are thought to be devoid of mast cells (Constantinides, 1953). Noguchi and Kandatsu (1976) have suggested that mast cells are a likely source of their enzyme.

Katanuma and his colleagues (Katanuma et al., 1975; Yasogawa et al., 1978; Sanada et al., 1978, 1979) also isolated a serine protease with an alkaline pH optimum (8.0) and a molecular weight of 22,000-24,000 from several rat tissues including muscle. However, the enzyme was shown to be very similar to the mast cell enzyme in many of its properties and was conclusively located in mast cells by immunofluorescent labelling (Woodbury et al., 1978a, 1978b). In previous studies these workers had shown increases in the enzyme's specific and total activity in mice with hereditary muscular dystrophy (Sanada et al., 1978). This apparently muscle specific response seems incompatible with a mast cell origin, however it may be explained by assuming that the number of mast cells remains constant in dystrophy and therefore during muscle atrophy the relative portion increases.

The cellular origin of an alkaline protease described by Mayer and his colleagues remains to be established (Mayer et al., 1974).

The enzyme has a pH optimum of 9.5, is inhibited by soybean trypsin inhibitor and is associated with the myofibrillar fraction. The enzyme has not been sufficiently characterized to permit useful comparison with the other proteases already described. However, an enzyme with similar properties has been prepared by Dahlmann and Reinauer (1978) who observed elevated alkaline proteolytic activity in muscles of diabetic rats (Röthig et al., 1978). On purification they found the enzyme had a molecular weight of about 31,000 a pH optimum of 9.4-9.6, similar bivalent ion requirements to Mayer's enzyme and was inhibited by soybean trypsin inhibitor. More significantly it was not affected by di-isopropyl phosphorofluoridate (DPF) or α -toluenesulphonyl (TPS) but was inhibited by p-chloromercuribenzoate (PCMB) indicating that it was a thiol rather than serine protease and therefore quite distinct from any of the previously described alkaline proteases. Mayer et al., (1974, 1976) investigated the significance of the enzyme in muscle catabolism under a variety of conditions. Under conditions of increased muscle proteolysis in vivo, such as starvation, diabetes, dystrophy and glucocorticoid administration the activity increased. On the basis of these studies it was suggested that the myofibrillar protease played a pivotal role in muscle protein catabolism and adapted in response to particular conditions. Interestingly laevodosin, a mixture of nucleotides and nucleosides, inhibited the enzyme and reduced its activity in vivo (Mayer et al., 1976).

Several alkaline proteolytic activities have been identified in

cardiac muscle. Both Bhan et al., (1978) and Murakami and Uchida (1978) have purified their respective enzymes and tested these against myofibrillar proteins. The results indicated that both were capable of selectively degrading the light chain 2 of myosin. Because of this specificity it was suggested that these enzymes could be involved in myosin turnover acting in a manner comparable to the "nick" scheme proposed for CAF. The cellular origins of these enzymes was not investigated but since mast cells are also found in the heart, it is possible that some of the cardiac enzymes are also derived from this source. Further investigations will be required to determine if skeletal or cardiac muscle contain an indigenous alkaline protease, and what role, if any, it may have in intracellular protein degradation.

b. Factors regulating protein degradation in muscle during states of growth and atrophy.

The capacity of mature skeletal muscle to alter its mass and composition in response to various stimuli including pathological stress, is well recognized. It is generally accepted that such changes, in the initial stages at least, do not involve changes in the number of muscle fibres, although in some states of growth increases in DNA content, presumably derived from satellite cell proliferation, have been observed (Kelly, 1978; Schultz, 1978). The mechanisms which underly these processes of growth (hypertrophy) and atrophy are not well understood, but it is apparent that regulation of cellular protein metabolism plays an important role (Young, 1970; Goldberg and St. John, 1976; Waterlow et al., 1978).

During steady state conditions the overall rates of protein synthesis and degradation are equal. However in states of growth or atrophy there is an imbalance in this equilibrium, leading to either a net accumulation or loss of protein. Theoretically, the transition from steady to non-steady state conditions could be induced by changes in either synthesis or degradation. Thus control of the rate of intracellular protein degradation is potentially as important as control of synthesis in regulating states of growth and atrophy in skeletal muscle, as well as other tissues. It should be emphasized that in this context protein degradation refers to the overall rate of proteolysis in the muscle and would therefore correspond to control at a coarse level. The rates of degradation of individual molecular species would depend upon both the level of adjustment of this coarse control and to specific fine control factors discussed previously.

Studies of normal growth in rats (Millward et al., 1976; Haverberg et al., 1978) and chickens, (Maruyama et al., 1978) have shown that the rates of both protein synthesis and degradation are initially high and gradually decline as growth proceeds, to lower levels characteristic of the adult. Decreased growth rates in young rats, induced by dietary restriction, were found to be primarily the result of decreased rates of protein synthesis while protein degradation remained more or less unaltered (Millward et al., 1976; Waterlow et al., 1978; Haverberg et al., 1978). Only during states of fasting or starvation when growth was either halted or atrophy actually induced, were increased rates of de-

gradation observed. In contrast to these results the decreased growth rates found in chicks fed diets deficient in both fuel molecules and amino acids, appeared to be mediated primarily through changes in the rates of degradation rather than synthesis (Maruyama et al., 1978). Since similar techniques were employed in both sets of studies it seems that methodological differences cannot explain the observed differences and the differences may simply reflect differences between species.

As mentioned above, only during states of fasting or starvation, were rates of protein breakdown found to be increased (Millward et al., 1976). Related studies of arterio-venous differences across the forearm of humans and the hind limb in rats, indicate that by 16 hours post-absorption there is a net release of amino acids from muscle (Felig, 1975). Such observations have led to the suggestion that under conditions of nutritional stress and in particular energy deprivation, skeletal muscle provides a source of amino acids which can serve as substrates for gluconeogenesis (Felig, 1975; Cahill, 1970). There is now a considerable body of evidence which suggests that part of this adaptive response may be regulated through the action of insulin. For example, several studies have shown that addition of insulin at physiological levels, to in vitro incubated skeletal and cardiac muscle, simultaneously stimulates protein synthesis and inhibits protein degradation (Goldberg, 1979; Jefferson et al., 1974; Fulks et al., 1975; Rannels et al., 1975; Lundholm and Scherstein, 1975, 1977). Increased synthetic rates induced by insulin, may be due to direct

influences such as stabilization of mRNA (Woll et al., 1972; Jefferson et al., 1974). Another possibility is that insulin induced enhancement of amino acid or glucose uptake stimulates protein synthesis since addition of these substances to the incubation medium, in the absence of insulin, were also found to increase synthesis. Interestingly, in the case of increased leucine concentration, the rates of protein degradation were also found to be decreased, but the mechanisms by which this occurs is presently unknown (Fulks et al., 1975; Buse and Weigand, 1977). Similarly the mechanism by which insulin causes a decrease in the rate of protein degradation is not known. However since this effect is also seen in the presence of inhibitors of protein synthesis such as cycloheximide it seems unlikely that decreased degradation occurs as a secondary response to changes in synthesis.

Another class of hormones which may be important in regulating muscle protein metabolism in certain conditions are glucocorticoids. During starvation glucocorticoid levels are elevated (Mayer and Rosen, 1977) and there is a marked decrease in the ability of adrenalectomized rats to maintain their glucose levels in response to fasting (Steel, 1975). This latter observation may reflect an inability in these animals to mobilize amino acids in peripheral tissues for conversion to glucose in the liver. In general the glucocorticoids are associated with marked catabolic effects in muscle, particularly when present in high concentrations as is found in Cushing's syndrome, but it is not certain whether this

is due to decreased protein synthesis or increased degradation. Goldberg (1969a) found by isotope decay methods, that the total activity in the muscle proteins of rats treated for 7 days with cortisone, was decreased with respect to untreated controls, while the specific activity was not altered. These results were interpreted as meaning that both decreased synthesis and increased degradation were responsible for the observed atrophy of the muscle. Shoji and Pennington (1977) also demonstrated that glucocorticoid treatment decreased protein synthesis in rat skeletal muscle, but were unable to find any evidence for increased catabolic rates. In fact after three days of steroid treatment decreased rates of protein degradation were found. More recently Goldberg et al., (1980) reported that fasting in adrenalectomized rats did not cause increased protein breakdown in their muscles in vitro, as was found for normal controls, indicating that that adrenal function perhaps through glucocorticoids, were required for stimulation of the tissue's protein degradation. The effects of glucocorticoid replacement in fasted adrenalectomized animals was not reported, therefore it is not absolutely clear that the failure to increase protein breakdown in these animals was due solely to the absence of glucocorticoids.

Mayer and his colleagues (Mayer and Rosen, 1977; Mayer et al., 1976) have reported that the catabolic effects of glucocorticoids on muscle, are paralleled by specific increases in the level of alkaline protease activity which they suggest may be a mechanism by which increased rates of proteolysis could be induced. However

as discussed in an earlier section, it remains to be established that the alkaline protease activity is in fact of muscle origin and not derived from mast cells present in the muscle tissue. Thus while the evidence for the effects of these hormones on protein synthesis in muscle is good, there is no agreement on their effects on protein degradation, and further studies will be required to clarify this point.

Hypophysectomy has also been used to suppress normal growth in young rats (Kostyo and Isaksson, 1977; Talwar et al., 1975). Under these conditions, Goldberg and his colleague found that both protein synthesis and degradation were decreased with respect to controls, in muscle isolated from these animals, (Goldberg et al., 1979, 1980). Treatment of hypophysectomized animals with growth hormone restored normal body growth and increased rates of muscle protein synthesis without affecting protein degradation (Goldberg et al., 1979, 1980). Earlier observations on in vitro incubated muscles had also shown that growth hormone did not affect rates of degradation (Goldberg, 1969b; Trenkle, 1974). The decrease in breakdown after hypophysectomy, was attributed to a lack of thyroid hormones (Griffin and Goldberg, 1977; Goldberg et al., 1977), since control levels of degradation could be restored by treatment with either tri-iodothyronine (T_3) or thyroxine (T_4), while thyroidectomy decreased breakdown. In addition the catabolic effects of large doses of T_3 or T_4 , characteristic of thyrotoxicosis, were correlated with significant increases in rates of proteolysis and the activities of several

lysosomal enzymes including cathepsin B and D (Goldberg et al., 1979; DeMartino and Goldberg, 1978). It was therefore suggested that thyroid hormones may act to increase protein catabolism in muscle by stimulating lysosomal activity, and that this may represent an important general mechanism in the regulation of muscle protein metabolism (Goldberg et al., 1979).

In addition to these nutritional and hormonal factors, several other factors have been found to be important in muscle growth and atrophy. Evidence from a number of sources has established that the level of mechanical activity in a muscle is an important influence on the growth state of the tissue (Laurent and Millward, 1980; Goldberg and St. John, 1976). In general, increased activity leads to muscle hypertrophy while decreased activity or disuse leads to atrophy. As with the growth states already discussed, these activity associated changes have been related to changes in the protein metabolism of muscle. To this end a number of experimental models have been developed (Laurent and Millward, 1980). Goldberg (1969b, 1971b) used tenotomy of the gastrocnemius to induce compensatory hypertrophy in the synergistic soleus, as a model for studying the relationship of protein metabolism to muscle growth. The results of these investigations suggested that during hypertrophy synthesis was increased while degradation was decreased with respect to contralateral controls. These changes led to significant increases in the weight and protein content of the tissue with sarcoplasmic protein increasing relatively more than myofibrillar. Laurent and Millward (1980) have argued that

the isotope decay measurements used by Goldberg in these studies, were probably in error due to extensive recycling of label and that increased protein synthesis in these muscles was sufficient to account for their growth. An interesting aspect of this work-induced growth is that it appears to be independent of hormonal factors and would therefore allow the organism to develop specific muscles in response to particular needs.

Using a different approach, Goldspink (1977a) investigated the effects of immobilization and stretch on protein metabolism in muscle. Immobilization atrophy was shown to correlate with decreased synthesis and increased degradation. Similarly Nihei and Sternberg (1978) and Walker and Strohman (1978) found that the half-life of myosin heavy chain in muscle cultures, was decreased whenever the spontaneous mechanical activity of the cultured cells were inhibited. In particular Walker and Strohman (1978) showed that there was no change in the rates of myosin heavy chain synthesis in inhibited cultures and they suggested that the decreased heavy chain half-life was caused by regulation at the post-translational level i.e. degradation. In contrast to these results Goldspink (1977a) also found that immobilized muscles held in a stretched position hypertrophied with respect to unrestrained controls. This stretch induced hypertrophy was apparently due to an increase in synthesis only, and it was suggested that both passive stretch and development of isometric tension induced through a stretch reflex, acted on the muscle in an unspecified manner to stimulate growth through protein synthesis.

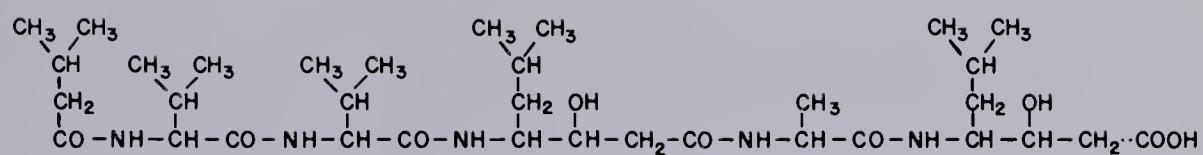
A related model of muscle hypertrophy is the denervated rat hemidiaphragm. In this case, although denervation eventually leads to atrophy, the initial response of the muscle is to hypertrophy (Turner and Garlick, 1974). This transient hypertrophy has been attributed to passive stretch of the denervated hemidiaphragm resulting from contractile activity in the innervated functional half. During the period of hypertrophy rates of protein synthesis were found to be increased as were rates of degradation (Turner and Garlick, 1974). Similar results were reported by Goldspink (1977b) for the denervated extensor digitorum longus muscle maintained in a fixed immobilized state. A difficulty of interpretation with these results is that the increased level of degradation could be the direct result of denervation (Goldspink, 1976, 1977b), rather than being part of the transient hypertrophy mechanism itself and in fact the rate of degradation could be the limiting factor which ultimately stops growth and induces atrophy. To some extent these difficulties can be overcome by the system developed by Laurent and Sparrow (1977), which involves attaching a suitable weight to the wing of the chicken and observing the resultant growth in specific muscles, usually the anterior latissimus dorsi (ALD). On the basis of studies in this model it was concluded that hypertrophy was accompanied by increased synthesis and degradation, with the increase in synthesis exceeding that for degradation (Laurent and Sparrow, 1977; Laurent et al., 1978; Laurent and Millward, 1980). The cellular mechanisms underlying these changes are presently unknown, but it has been suggested that

stretch or stretch induced isometric tension, may result in muscle plasma membrane alterations which are prerequisite to the changes in protein metabolism (Laurent and Millward, 1980). Thus, increased uptake of amino acids observed in stretched muscles could lead to secondary stimulation of protein synthesis (Nahara and Holloszy, 1974; Goldberg et al., 1975). As for the relationship of stretch in intracellular protein degradation, it is not possible to draw meaningful conclusions until details of the individual steps in degradation are elucidated. All that can be said at present is that the behavior of degradation during states of growth and atrophy in muscle appears to depend very much on the nature of the precise inducing stimulus.

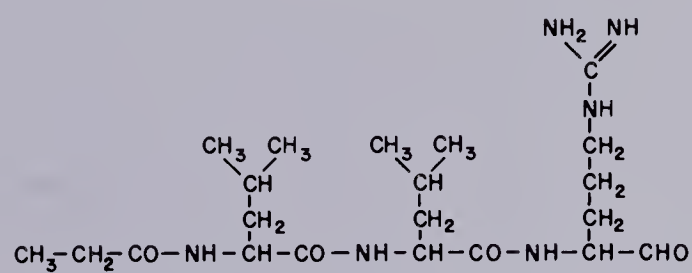
D. The Microbial Protease Inhibitors

Leupeptin and pepstatin are peptides which belong to a group of naturally occurring, low molecular weight proteinase inhibitors. They were first described by Umezawa and his coworkers (Umezawa, 1972, 1976) who discovered them during screening studies of filtrates of actinomycetes cultures. These workers had reasoned that microbial cells such as actinomycetes, which were thought to obtain nutrients from their environment by secreting proteases to digest organic nitrogen, may also produce protease inhibitors as a self-protection mechanism against the potentially harmful effects of the proteases. The structures of leupeptin and pepstatin are shown in Figure 2, and some of their properties are listed in

Figure 2. Molecular structure of leupeptin and
and pepstatin (Umezawa, 1976).



PEPSTATIN



LEUPEPTIN

TABLE 3
PROPERTIES OF LEUPEPTIN AND PEPSTATIN

INHIBITOR	MOL. WT.	SOLUBILITY	ID ₅₀ (ug/ml)	SPECIFICITY
Leupeptin	482	H ₂ O, polar organic solvents, DMSO	a) 0.44 (cathepsin B) b) 2.00 (trypsin) c) 0.50 (papain)	Relatively broad for both serine and thiol dependent proteinases.
Pepstatin	686	polar organic solvents, DMSO, low solubility in H ₂ O	a) 0.11 (cathepsin D) b) 0.0031 (pepsin)	carboxyl proteinases

Data from Umezawa (1976).

Table 3.

Structural studies (Umezawa, 1972) revealed that leupeptin was in fact a mixture of several compounds, two major forms of which were acetyl-L-leucyl-L-leucyl-L-arginal (leupeptin-Ac-LL) and propionyl-L-leucyl-L-leucyl-L-arginal (leupeptin-Pr-LL). The occurrence of other less common forms, in which either or both of the leucine residues had been substituted with valine or isoleucine, was also reported. In a survey of its inhibitory properties (Ayoagi et al., 1969), leupeptin was tested against a number of different proteases, for its ability to inhibit both proteolytic and esterolytic activities. It was found to be a strong inhibitor of the proteolytic effects of papain, trypsin and plasmin, a somewhat weaker inhibitor of thrombokinese (thromboplastin) and was ineffective against α -chymotrypsin at concentrations greater than 500 ug/ml (the maximum concentration tested). Its inhibition of esterolysis by plasmin, trypsin and kallikrein was in general found to be considerably weaker and in the case of thrombin virtually non-existent. More recently it has been shown that leupeptin is an effective inhibitor of a thiol dependent Ca^{++} - activated neutral proteinase from skeletal muscle (Libby, 1977, personal communication). On the other hand leupeptin does not inhibit collagenase, a metalloendoproteinase, from polymorphonuclear leucocyte granules (Barrett, 1974; Wingender, 1974). Of several other tissue proteinases examined only cathepsin B, a thiol proteinase similar to papain was inhibited (Barrett, 1974; Wingender, 1974). Cathepsins C, D and E and elastase were not

inhibited. Thus leupeptin does not appear to be specific for a particular class of proteinases nor does it inhibit all proteinases within any given class.

Preliminary studies on its mechanism of action (Umezawa, 1972, 1976) indicated that the arginal residue was essential to its inhibitory activity and furthermore that the aldehyde group of this residue was important. These observations led to the suggestion that leupeptin acted in a manner similar to synthetic peptide aldehyde inhibitors of both serine and thiol proteinases (Barrett, 1974). These inhibitors were believed to form stable covalent hemiacetal (with serine proteinases) and thiohemiacetal (with thiol proteinases) addition complexes analagous to the postulated tetrahedral intermediates of the catalytic mechanism. A recent crystallographic and kinetic study (Brayer et al., 1979) of the interaction of N-acetylprolylalaninyl-prolylphenylalaninal with the serine protease SGPA, clearly demonstrated the formation of a tetrahedral addition complex and confirmed the covalent nature of the link. Thus it seems likely that all of these peptide aldehydes including leupeptin act in a similar manner to inhibit both serine and thiol proteinases. The fact that leupeptin does not inhibit all proteinases from these two classes may be explained in terms of specific structural properties which render it incompatible for binding to the active site, in some cases.

As with leupeptin, pepstatin was found to be a mixture of structurally related compounds differing mainly in the nature of a fatty acid residue linked at the N-terminal by a peptide bond

(Umezawa, 1972, 1976). In the example shown in Figure 3 the fatty acid moiety is isovaleric acid but samples containing other fatty acids such as caproic, isocaproic and heptanoic acid have also been described. Peptide structure and amino acid analysis (Morishima et al., 1970) revealed the presence of one alanine and two valine residues along with two residues of an unusual amino acid, 4-amino-3-hydroxy-6-methyl-heptanoic acid (statine). Studies of its inhibitory properties showed that pepstatin was a specific potent inhibitor of acid proteinases including pepsin, renin and cathepsins D and E (Barrett, 1974; Guyene et al., 1976). One exception, however, was the acid proteinase A from Aspergillus niger, although having an acid pH optimum, is believed to be catalytically distinct from other acid proteinases.

Preliminary studies on the mechanism of action of pepstatin on pepsin (Marciniszyn et al., 1977) showed that while esterification of the carboxyl terminal had no effect, acetylation of the hydroxyl groups of the statine residues drastically reduced its inhibitory activity. The importance of the statine residues was further underlined when it was demonstrated that only fragments of pepstatin containing statine possessed inhibitory activity and that N-acetyl-statine itself was a weak competitive inhibitor (K_i $1.4 \times 10^{-4}M$, against globin). The manner by which the statine interact with the acid proteinases is at present unknown, but it has been shown in one case at least (Subramanian et al., 1977), that pepstatin binds in a cleft of the acid proteinase from Rhizopus chinensis, and the geometry of this cleft is very similar

to that identified as the active site in penicillopepsin (Hsu et al., 1977; James et al., 1977). In addition it could be seen that the bound pepstatin was situated very close to a catalytically active aspartate residue suggesting this as a possible mode of inhibition. Further studies will be required to elucidate the details of the interaction and consequent inhibition but it seems probable that pepstatin is structurally adapted to inhibition of a catalytic mechanism common to all carboxyl proteinases (Table 2).

II. STATEMENT OF THE PROBLEM AND RELEVANCE OF STUDY

It will be apparent from the foregoing discussion that there is ample evidence indicating that skeletal muscle proteins in general and the myofibrillar proteins in particular, like the proteins of other tissues, exist in a state of continuous turnover. However, the mechanism by which these proteins are degraded during turnover either in terms of specific steps in breakdown pathways or the enzymology involved is at present uncertain. In addition, it is not known whether the relative increase in muscle protein breakdown observed in dystrophic animals is the result of increased activity of normally functional mechanisms or processes unique to the pathological state. In an attempt to resolve these controversies the following studies were undertaken,

- a) The kinetics of protein degradation in normal and dystrophic chick skeletal muscle in culture were examined and compared.
- b) The kinetics of myofibrillar turnover in the same system were examined.
- c) The effects of the microbial protease inhibitors leupeptin and pepstatin on these processes were investigated and the susceptibility of normal and dystrophic muscles to these agents was determined.

It was hoped that information from the study would be of value to both Agricultural and Medical Scientists. Thus in areas of animal science where the growth and development of muscle is important, studies of this nature could contribute meaningfully.

In addition studies of pathological status in muscle could lead to a better understanding of the underlying processes and perhaps contribute to treatment of such diseases.

III. MATERIALS AND METHODS

A. Materials

Leupeptin was purchased from the Protein Research Foundation (Tokyo, Japan) and pepstatin was a gift from Professor H. Umezawa of the Institute of Microbial Chemistry, Tokyo, Japan. Sulphur-35 labelled methionine was obtained from the New England Nuclear Corp., Boston, U.S.A. The specific activity of the labelled methionine varied from batch to batch but fell in the range, 800 Ci/mmole to 1200 Ci/mmole. NCS tissue solubilizer was obtained from Amersham Corporation, Ontario, Canada. Bicarbonate-free Waymouths medium, powdered bicarbonate-free Hanks balanced salts, penicillin/streptomycin mixture and fungizone, for use in tissue culture were obtained from GIBCO, Canada, Calgary, Alberta. Horse serum was purchased from Flow Laboratories, California, U.S.A. Rooster serum was prepared from the blood of adult White Leghorn roosters maintained by the Poultry Division of the University of Alberta Farm, Edmonton, Alberta, Canada. Nitex nylon mesh was obtained from Mills Corp., Ontario, Canada, and Corning plastic tissue culture dishes were purchased from GIBCO, Canada, Calgary, Alberta. Acrylamide for use in gel electrophoresis was obtained from Bio-Rad Laboratories or Serva Chemical Co., New Jersey, U.S.A. All other reagents for electrophoresis were purchased from Bio-Rad Laboratories, Mississauga, Ontario, Canada. Coomassie-G-250 for use in the dye binding protein assay was purchased from the Eastman Kodak Company. Nonidet-P-40 was a gift from Professor J. Charnock of the Department of Pharmacology, University of Alberta,

Edmonton, Alberta, Canada. Collagenase Type II was obtained from the Worthington Biochemical Co., U.S.A. Purified collagenase (Worthington CLSPA) was supplied by Dr. H. Pearson of the faculty of Dentistry, University of Alberta, Edmonton, Alberta, Canada. All other chemicals were obtained from either Sigma Chemical Company, St. Louis, Mo., U.S.A. or Fisher Scientific Company, Edmonton, Alberta, Canada.

B. Chick Embryos

Fertile eggs from White Leghorn chickens were obtained from the Poultry Division of the University of Alberta Farm, Edmonton, Alberta, Canada. Fertile eggs from genetically dystrophic chickens carrying the mutation am/am (Asmundson and Julian, 1956) were obtained from Dr. P. Holland of the Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. The following information on the dystrophic flock was kindly supplied by Dr. P. Holland. The dystrophic flock, which consisted of 80 hens and 20 roosters, was obtained directly, by Dr. E. Cosmos of McMaster University, Hamilton, Ontario, Canada, from the Department of Animal Genetics, University of Connecticut, Storrs, Connecticut, U.S.A. The birds were maintained in individual laying cages and insemination of hens was carried out artificially. Since the inbreeding of such a small flock could give rise to problems of selection of traits not related to muscular dystrophy, only embryos which were the immediate offspring of the original Storrs flock, were used in the present study.

C. Cultures

Identical procedures were used in the preparation of cultures from both normal and dystrophic chick embryos. Primary cultures of chick embryo skeletal muscle were prepared by a modification of the method of Bullaro and Brookman (1976). Freshly dissected leg muscle from 6 or 7, eleven-day embryos were minced finely with scissors, suspended in growth medium (5ml) and transferred to a sterile, capped test-tube (25 x 125 mm). The mince was then dissociated by vortexing at full speed for three separate intervals of 15 seconds and the resulting cell suspension diluted with growth medium (10ml). After standing for 10 minutes at room temperature the upper two-thirds was drawn off and filtered consecutively through a 75- and 15- micron pore size Nitex nylon mesh. The filtered suspension was diluted with an additional 10 ml. of growth medium and preplated on non-gelatinized tissue culture plates (90mm) for 15 minutes at 37°C in an atmosphere of 5% CO₂. The cell suspensions were then drawn off, pooled, counted on a hemocytometer and plated on gelatin coated plates (Hauschka, 1972) at a density of either 3.0×10^6 cells/90 mm plate or 1.0×10^6 cells/60 mm plate. The cells were incubated at 37°C in an atmosphere of 5% CO₂.

Growth medium was a modification of Colemans NC medium (Coleman et al., 1978) and consisted of 40:60 Hanks balanced salt solution : Waymouths medium, 10% selected horse serum, 1% rooster serum, 1% antibiotic mixture and 0.25% fungizone. The bicarbonate concentration was adjusted to give a final concentration of

21.0 mM. Individual batches of horse serum were screened for their ability to support myogenesis and maintain healthy, cross-striated myotubes for periods of twelve days before selection for use in experiments. Rooster serum was prepared as follows. Blood was withdrawn from the wing vein of the roosters and under aseptic conditions transferred to sterile glass tubes and incubated at 37°C for 1h. It was then held overnight in the fridge and subsequently the clot was spun down at low speed, the serum pipetted off, sterilized by filtration and stored at -70°C. Gelatin solutions for coating culture plates were prepared as described by Hauschka (1972).

Photography of the cultures was carried out using either an Olympus CK or Nikon M phase contrast inverted optics microscope. Kodak Tri-X black and white film (ASA 400) was used for this purpose. Cultures were placed on the microscope stage and photographed using the automatic shutter control at predetermined settings. A green filter was used for all photographic procedures.

D. Experimental Protocols

Radioisotopic labelling conditions and addition of the protease inhibitors to the cultures varied depending upon the particular experiment.

In experiments to determine the effects of the inhibitors on protein degradation in the extractable cell protein fraction (ECP) cultures were grown on 60 mm plates for 4 days, at which time the medium was changed and replaced with fresh growth medium

(2 ml) containing L-(³⁵S)-methionine (1.8 uCi/ml). Twenty-four hours later the labelling medium was removed and the cultures washed three times with Hanks balanced salt solution (3.5 ml /60mm plate/wash). Cultures (0 hour samples) were then extracted for cell proteins as described below or were returned to the incubator with fresh growth medium (3.5 ml) containing unlabelled L-methionine at a final concentration of 2 mM. Where appropriate i.e. in cultures chosen for specific treatments, protease inhibitors at the concentrations indicated (see Results section) and/or dimethyl sulphoxide (DMSO) at a final concentration of 0.5%, were included in the medium. Samples of controls and treatments from each set of cultures were taken at 24 and 72 hours post-labelling, extracted for cell proteins and analyzed for TCA precipitable radioactivity as described below. An identical procedure was used in the determination of the dose/response relationship with the exception that the experiment was terminated 24 hours post-labelling and various concentrations of inhibitors were used.

In experiments to determine the effects of the protease inhibitors on the incorporation of labelled methionine into the ECP fraction, cultures were grown on 60 mm plates for 4 days at which time the medium was changed and replaced with fresh growth medium (3.5 ml/plate). Protease inhibitors and/or DMSO at the concentrations indicated were added to the media of treatment cultures at this stage. Twenty-four hours later the medium was replaced with fresh growth medium (2 ml) containing appropriate addition of inhibitors and/or DMSO and L-(³⁵S)-methionine

(0.9 uCi/ml). After 12 h incubation, the labelling media were removed, the cultures washed three times with Hanks balanced salt solution (3.5 ml/60 mm plate/wash) and extracted for ECP and analyzed for TCA precipitable radioactivity (see below).

In experiments to determine the effects of the protease inhibitors on myofibrillar turnover, cultures were grown on 90 mm plates. Four days after plating, the medium was changed and replaced with fresh growth medium (10 ml). Two days later (6th day of culture) the medium was changed again and replaced with fresh growth medium (5 ml) containing cytosine arabinoside (5×10^{-6} M) and L-(35 S)-methionine (4.5 uCi/ml). Twenty-four hours later the labelling medium was removed and the cultures washed three times with Hanks balanced salt solution (10 ml /90 mm plate/wash). Cultures (0 hour samples) were then used for the isolation of myofibrils as described below, or returned to the incubator with fresh growth medium (10 ml) containing unlabelled L-methionine (2 mM) and DMSO (0.5%). The addition of protease inhibitors to the medium of treatment cultures was initiated at this stage. Control and treatment cultures were taken at 24 and 72 hours post-labelling and myofibrils were isolated and analyzed as described below.

In all experiments leupeptin and pepstatin stock solutions (2 mg/ml) were prepared fresh for each set of cultures. Leupeptin was dissolved in water (10 ml) and sterilized by filtration through a 0.22 um millipore filter. Pepstatin (20 mg) was suspended in water by vigorous mixing for 15 minutes, titrated with one drop of

1.0 N NaOH and made up to volume. The cloudy suspension cleared almost instantaneously on addition of base, and the solution of pepstatin was sterilized as for leupeptin. Aliquots of these stock solutions were added to growth medium containing DMSO (0.5%) to give the final concentrations indicated for individual experiments.

E. Analysis of Extractable Cell Protein (Determination of Trichloroacetic Acid Precipitable Radioactivity)

Extracts of cell proteins were prepared in a manner similar to that described by Devlin and Emmerson (1978). Cultures were washed as described previously, thoroughly drained and extracted with 2 ml /60 mm plate of SNU buffer (0.2% SDS, 2% Nonidet-P-40, 9.5 M urea). The plates were gently agitated for 10 minutes, allowed to stand a further 20 minutes and the protein extracts were then transferred to Eppendorf tubes and centrifuged on a Eppendorf 3200 microcentrifuge for 4 minutes. Aliquots (50 μ l) of the centrifuged extracts were incubated in 0.3 N NaOH (1 ml) for 20 minutes at room temperature and then in 15% TCA (5ml) for 20 minutes at 4°C. The protein precipitates were collected on Whatman GF/A glass filters and dried in an oven at 50°C for 2 hours. The precipitates were then dissolved by incubating in 90% NCS (1 ml) for 2 hours at 55°C. Scintillation cocktail was added and the samples counted in the Beckman LS-330 liquid scintillation counter. The cocktail used throughout this study was a standard PPO-POPOP-ethylene glycol monomethyl ether-toluene mixture.

Protein concentrations of the cell extracts were determined by a modification of the dye binding assay of Bradford (1976) using bovine serum albumin (BSA) as a standard. Cell extracts (100 μ l) were diluted ten-fold with water and aliquots (50 μ l) of the diluted extract were mixed with Bradford reagent (1.5 ml). Fifteen minutes after mixing the optical density of the resulting solution was read at 595 nm and the protein concentration determined by comparison with the standard curve. Protein determinations were performed in triplicate for each sample and for BSA standards. In preparation of the standard curves, SNU diluted to one-tenth of its original concentration was used as solvent for the BSA standards. Aliquots (50 μ l) of these standard solutions were mixed with Bradford reagent (1.5 ml) and the optical density determined as for the cell extracts. This procedure was used in order to ensure that the final concentration of SNU was the same in both standards and cell extracts. In addition blanks containing only SNU at the appropriate concentrations showed only a slight increase in optical density (0.025-0.030 optical density units) with respect to the Bradford reagent, indicating that at the concentrations used, SNU did not interfere with the assay. Bradford reagent was prepared as follows. Coomassie-G-250 (100 mg) was dissolved in 95% ethanol (50 ml) and diluted with 85% phosphoric acid (100 ml). Deionized, distilled water (150 ml) was added and the solution was filtered (Whatman #1). The filtrate was adjusted to a volume of one liter with deionized distilled water. New standard curves were determined for each preparation of reagent.

F. Isolation of Myofibrils

Only cultures which exhibited cross-striations and spontaneous contractions were used initially, although the procedure was subsequently tested on less developed cultures. All steps were carried out at 0°-4° C unless otherwise stated.

Medium was first decanted and the plates washed three times with Hanks BSS, pH 7.1. Each plate was then treated with 5.0 mM KCl, 2.0 mM ethylenediaminetetraacetic acid (EDTA), 10mM Tris-HCl pH 7.6 (10 ml /90 mm plate) for 5 minutes, after which time the buffer was renewed and the plates allowed to stand an additional 15 minutes. The relaxed myotubes were then extracted with rigor inducing buffer (RIB) containing 2% Triton-X-100, 2.0 mM EDTA, 0.1 M KCl, 0.5 mM dithiothreitol (DTT), 10 mM Tris-HCl pH 7.2 (10 ml /90 mm plate), for two consecutive periods of 5 and 10 minutes. After decanting the RIB the plates were washed twice with Ca^{++} and Mg^{++} free BSS and the rigor cells were detached by brief exposure to 0.01% collagenase, 0.005% DNAase at room temperature for 5 minutes. The detached material was immediately sedimented at low speed resuspended by vortexing for 15 seconds in RIB (2 ml) and homogenized by three passages through a 3-inch, 22-gauge cannula. After standing on ice for 10 minutes the upper three-quarters was carefully withdrawn and retained, and the settled material homogenized a second time (10 passes) after resuspension in RIB (1.5 ml). The pooled supernatants were sedimented at 800 x g for 5 minutes and the pellet containing mostly myofibrils

washed three times with RIB. Sodium deoxycholate (DOC) at a concentration of 0.02% was added to the last wash to assist in extraction of residual membranous materials. The myofibril pellet obtained from the last wash was prepared for electrophoresis by dissolving in 500 μ l of 8 M urea, 2.5% SDS, 5 mM EDTA, 5 mM DTT, 100 mM Tris/glycine pH 8.80 at 100°C for 5 minutes. Glycerol was added to a final concentration of 30% (v/v) and two drops of a concentrated solution of bromophenol blue (0.05%) added as a tracking dye.

G. Electrophoresis

Electrophoresis of samples and standard proteins was carried out on a Buchler Polyanalyst tube gel system using an Ortec constant voltage power supply. Samples and standards were run on 10% polyacrylamide gels crosslinked with 0.1% bis-acrylamide, in the presence of sodium dodecyl sulphate at a current of 3.0 mA/tube. Each run lasted about 2 hours and was terminated when the tracking dye was approximately 1 cm from the bottom of the gel. The gels were then removed from the tubes and placed in 25% isopropanol/10% acetic acid fixative. After 2 hours the fixative was decanted and replaced by 0.01% Coomassie brilliant R-250 blue stain solution, and the gels allowed to stand overnight. Initial destaining was done electrically for 25 minutes in 7% acetic acid using a Canalco quick gel destainer. This was followed by diffusion destaining in 5% methanol/10% acetic acid lasting several days until the backgrounds were clear. Destained gels were quantified by scanning and densitometry at 550 nm in a

Gelman Automatic Gel Scanner, and sliced into uniform 1 mm segments using a Bio-Rad model 195 Electric gel slicer. The sliced gel was then placed on a light box and regions corresponding to complete resolved bands were teased free. In addition a single segment corresponding to the point of lowest optical density on each gel was removed and used as background. The segments for each band were placed in scintillation vials with a maximum of 3 slices per vial, and 90% NCS (1 ml) was added to each vial. The samples were tightly capped and incubated at 55°C overnight. Standard cocktail was then added and the samples counted in a Beckman LS-330 liquid scintillation counter. Peak areas of the densitometric scans were quantified by the method of triangulation.

H. Statistical Analysis

Data for a single experiment constitutes the results obtained from one set of cultures i.e. a single cell preparation. Within each set of cultures, controls and treatments (on a per plate basis) were established in triplicate or quadruplicate. The value of any variable e.g. TCA precipitable counts, was determined for each plate and the arithmetic means, standard deviations and standard errors of the means were determined for each set of triplicates or quadruplicates. Analysis of variance was performed using the Students-t-test to compare the means of both treatments and controls, and different treatments. Similarly mean values from repeats of individual experiments were used to obtain a second set

of means, standard deviations and standard errors of the means, which were also analyzed for variance using the Students-t-test. Calculated data e.g. % inhibition, obtained from the measurements of experimental variables were analyzed in the same way. If statistical probability (p) was less than 0.05 the means were considered significantly different.

Linear regression analysis was performed by computer using the method of least squares to obtain best fits of the semi-logarithmic plots of specific activity versus time for each of the myofibrillar components, in individual experiments. Slopes and correlation co-efficients were determined. The significance level of the correlation co-efficient (R) was also computed using the Students-t-test with $n-2$ degrees of freedom.

The following calculated data were obtained from primary measurements.

1) Percent Inhibition

This value was obtained by subtracting either total counts or specific activity of 24 and 72 hour control values from the corresponding treatment value and expressing this difference as a percentage of the difference between the 0 hour value and the controls at either 24 or 72 hours.

2) Average Rates of Decay

This value was obtained for each curve in the two intervals 0-24 and 24-72 hours. The difference in counts per minute between the start and finish of each

interval was divided by the time elapsed (hours) during the interval.

3) Half-Lives

Half-lives were obtained by substitution of the k_d value obtained from linear regression analysis, into the equation

$$t_{1/2} = \frac{\ln 2}{k_d}$$

where $t_{1/2}$ = half life

k_d = rate constant of degradation.

IV. RESULTS

A. Morphological Observations

Under standardized conditions, skeletal (leg) muscle cultures derived from normal chick embryos, underwent a reproducible sequence of developmental changes (Figure 3a-3d). During the first 24 hours in culture, many of the cells were seen to attach to the substratum and begin dividing (Figure 3a). Most of the cells present at this stage were tentatively identified as myoblasts on the basis of their characteristic spindle shape (Konigsberg, 1971). This period of cell division continued until about 40 hours post plating, at which time the myoblasts began to align and fuse, forming multinucleated syncytia (Figure 3b). By 72 hours the cell fusion was essentially complete and the cultures consisted of an extensive network of large multinucleated myotubes (Figure 3c). Further development resulted in continued hypertrophy of the myotubes, the appearance of distinct cross-striations, and the initiation of spontaneous twitches on day 6 or 7. Twitches were often seen prior to the appearance of cross-striations suggesting that a functional contractile apparatus was present within the myotubes even though this was not morphologically evident. Contamination of the cultures by non-myogenic cells (mostly fibroblasts) was in general fairly low during the first 4 or 5 days, becoming more apparent after this time.

The development of cultures prepared from the leg muscle of genetically dystrophic embryos was essentially identical in both morphology and time scale (Figure 4a-4d). Although not striking,



Figure 3. Morphological Development of Normal Chick Embryo Leg Muscle in Culture. Cultures (1×10^6 cells/60 mm plate) were established as described in Materials and Methods and grown for 8 days. Cultures were taken at a) 24 hours, b) 48 hours, c) 72 hours, d) 8 days, representative fields selected and photographed.

NB. 8-day old cultures were rinsed once with RIB before photography in order to illustrate cross-striation more clearly. Calibration bar 50 μ m.

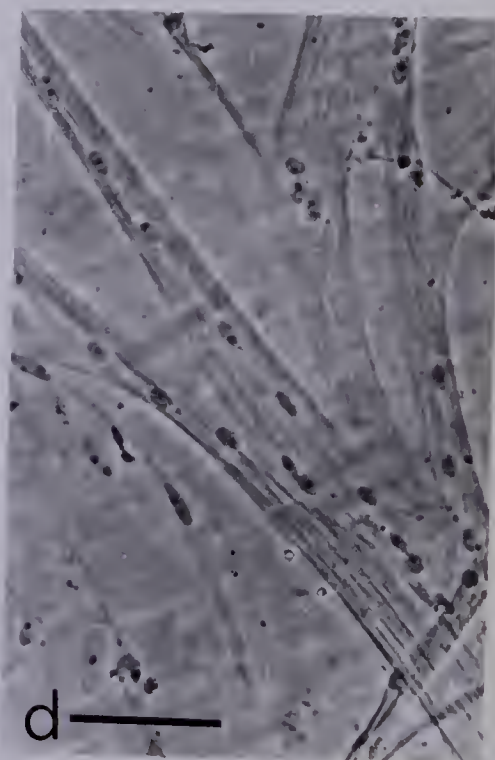
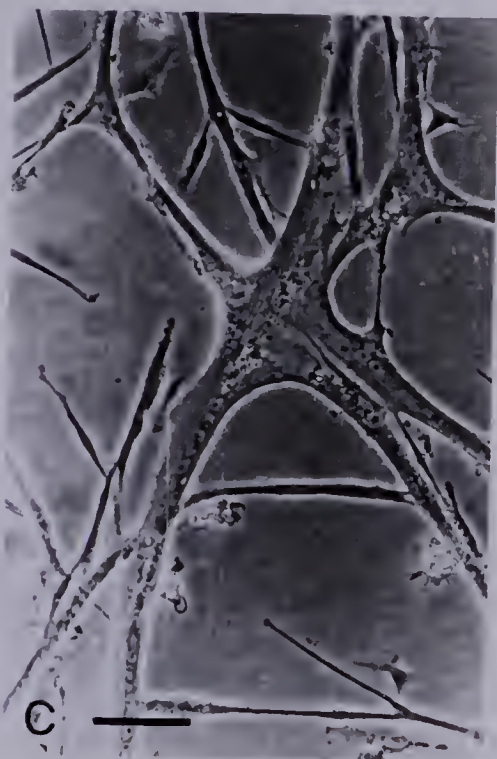


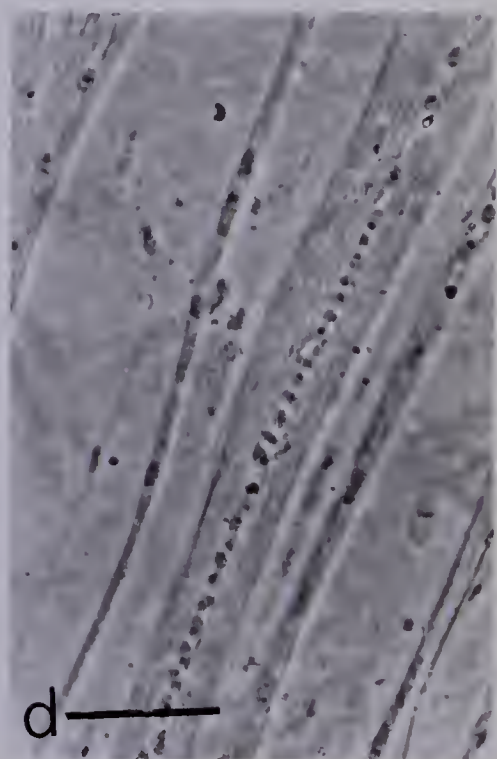
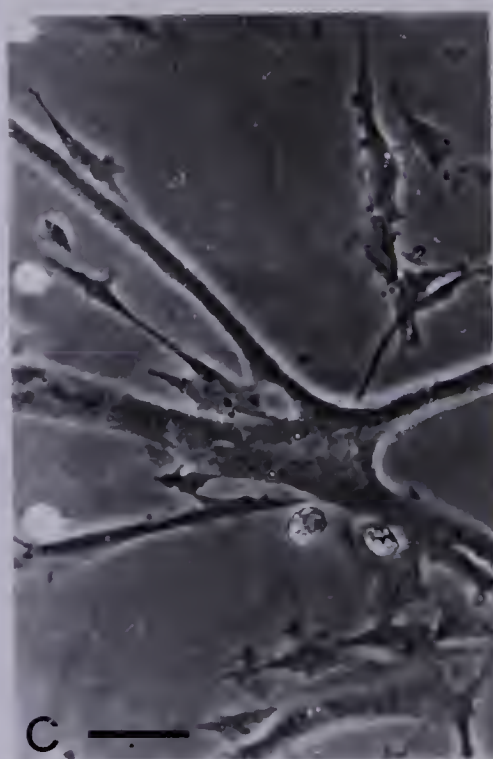


Figure 4. Morphological Development of Dystrophic
Chick Embryo Leg Muscle in Culture.

Cultures (1×10^6 cells/60 mm plate) were
established as described in Materials
and Methods and grown for 8 days.

Cultures were taken at a) 24 hours,
b) 48 hours, c) 72 hours, d) 8 days,
representative fields selected and
photographed.

NB. 8-day old cultures were rinsed once
with RIB before photography in order to
illustrate cross-striation more clearly.
Calibration bar 50 μm .



it did however appear that the level of fibroblast contamination was slightly greater in dystrophic cultures, particularly after the 6th or 7th day of culture.

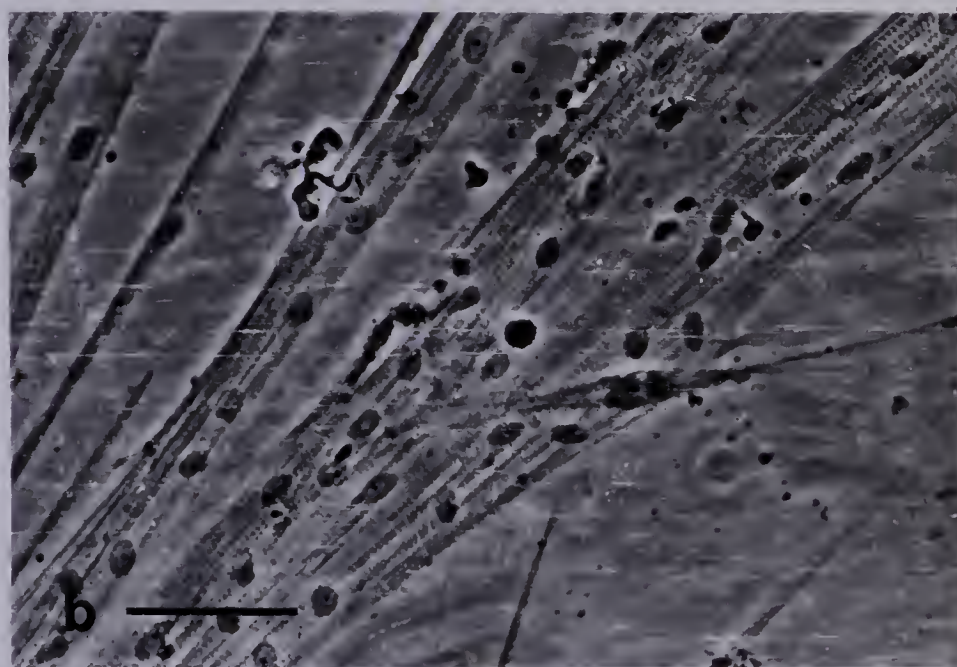
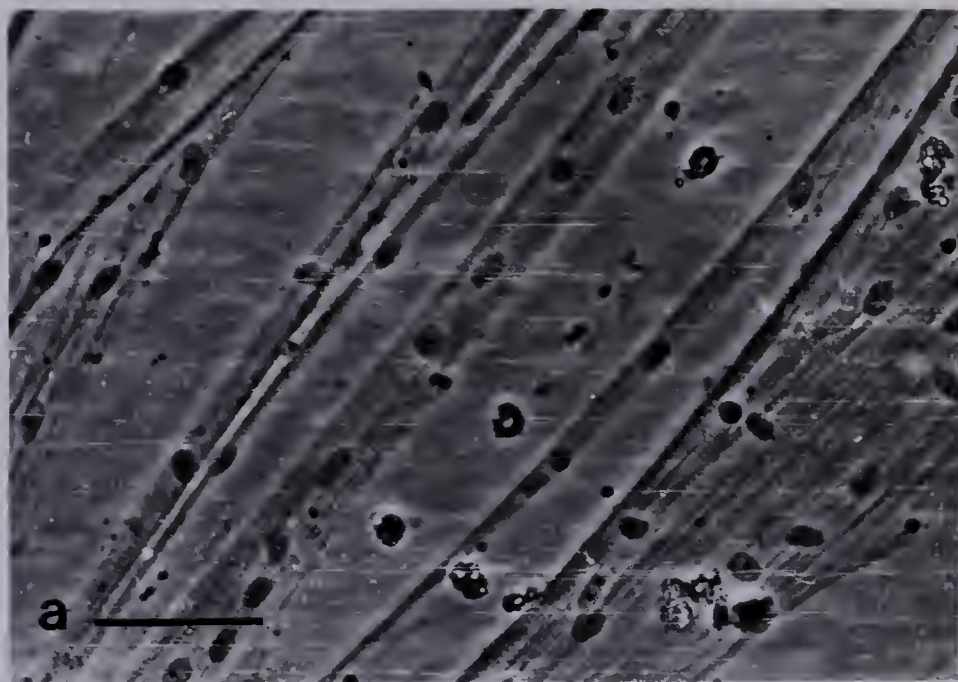
Treatment of normal cultures with leupeptin (50 ug/ml.), pepstatin (50 ug/ml.) or a combination of both inhibitors (50 ug/ml.each), produced no obvious morphological changes (Figure 5). Similar results were obtained from dystrophic cultures, treated during the same interval in culture, with the exception that the combined use of the inhibitors for up to 72 hours appeared to cause the myotubes to develop a granular appearance (Figure 6). (This may also have occurred in the leupeptin sample but was not as obvious.) This effect was much more noticeable in older dystrophic cultures treated with the combination of inhibitors between the 7th and 10th days in culture (Figure 7). In these cases the effect began to be noticeable about 48 hours after addition of the inhibitors to the cultures and progressed to a state of extensive degeneration by 72 hours of exposure (Figure 7). The nature of the granular material was not established but appeared similar to granular material observed in other cell types in culture and identified as autophagic vesicles or lysosomes (Smith and Van Frank, 1975).

B. Analysis of Extraction Cell Protein

With regard to the efficiency of extraction of cultures by SNU buffer two points can be made. Firstly, microscopic examination of cultures during extraction revealed that in the presence of SNU there was a very rapid and virtually complete loss of



Figure 5. Effect of protease inhibitors on the morphology of cultures of normal chick embryo leg muscle. Cultures (1×10^6 cells/60 mm plate) were established as described in Materials and Methods and grown for 5 days with one change of medium on day 4. Treatment with leupeptin (50 ug/ml) and pepstatin (50 ug/ml) was initiated on day 5 and maintained over the following 72 hours. Cultures were then removed from the incubator, rinsed once with RIB representative fields selected and photographed. a) controls, b) 72 hour leupeptin, c) 72 hour pepstatin, d) 72 hour leupeptin and pepstatin. Calibration bar, 50 um.



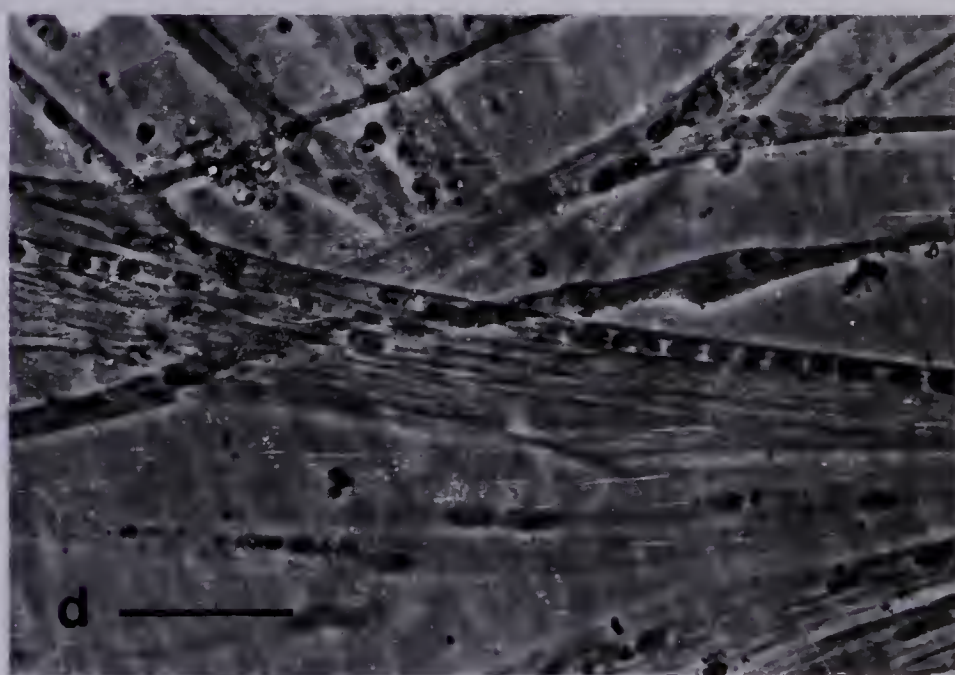
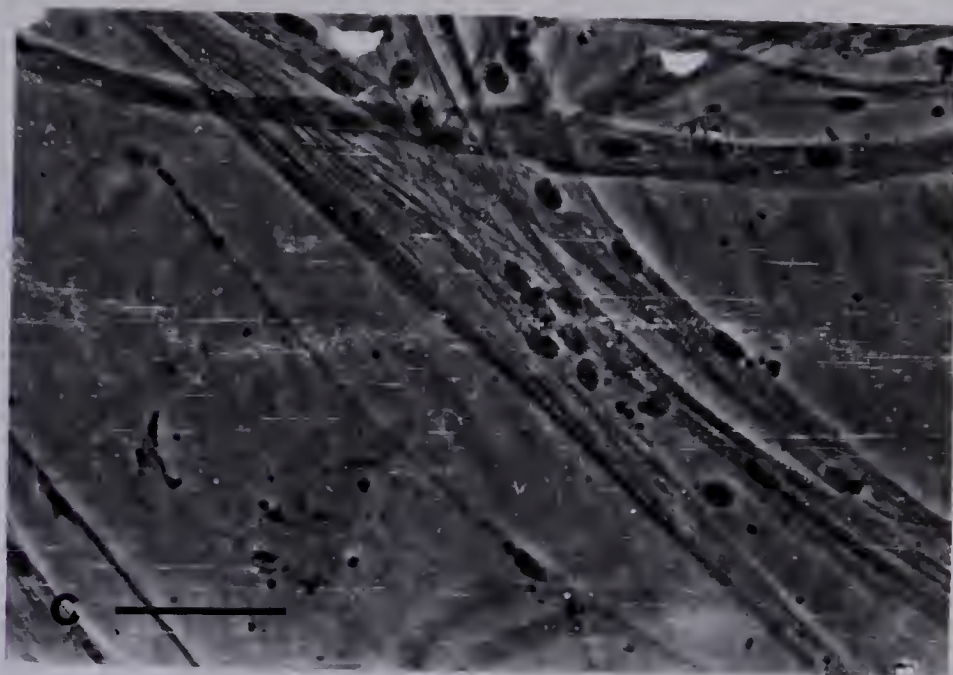




Figure 1. The effect of the concentration of the solution on the rate of the reaction.

The rate of the reaction was measured by the change in the concentration of the reactants over time. The results are shown in the following table:

Concentration of the solution (M)	Rate of the reaction (M/s)
0.1	0.01
0.2	0.02
0.3	0.03
0.4	0.04
0.5	0.05

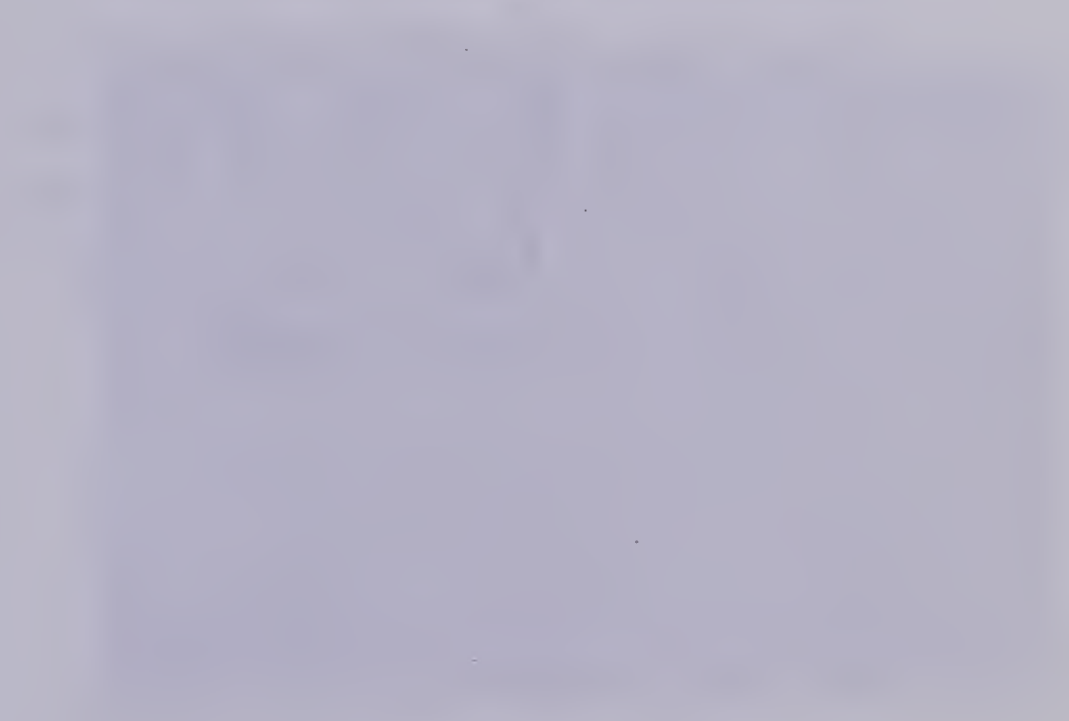
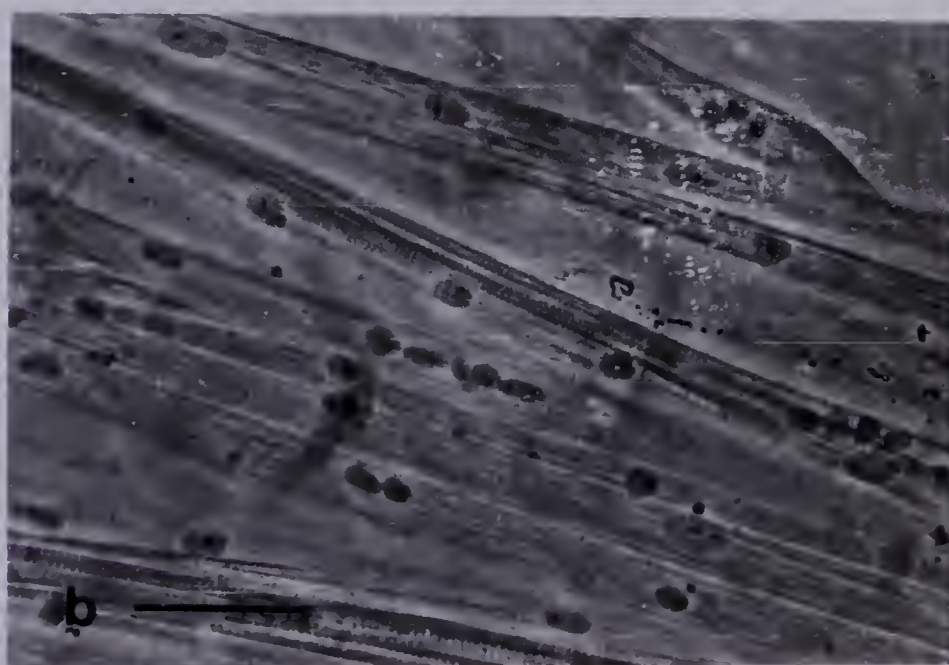
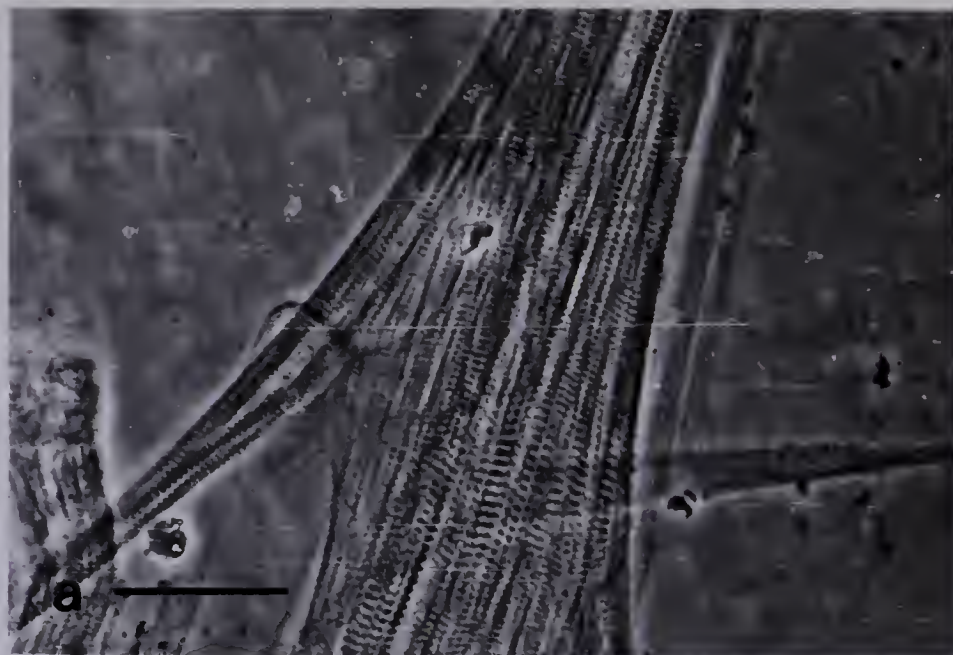


Figure 6. Effect of protease inhibitors on the morphology of cultures of dystrophic chick embryo leg muscle. Cultures (1×10^6 cells/60 mm plate) were established as described in Materials and Methods and grown for 5 days with one change of medium on Day 4. Treatment with leupeptin (50 ug/ml) and pepstatin (50 ug/ml) was initiated on Day 5 and was maintained over the following 72 hours. Cultures were then removed from the incubator, rinsed once with RIB representative fields selected and photographed. a) control b) 72 hour pepstatin c) 72 hour leupeptin d) 72 hour leupeptin + pepstatin. Calibration bar 50 mm.



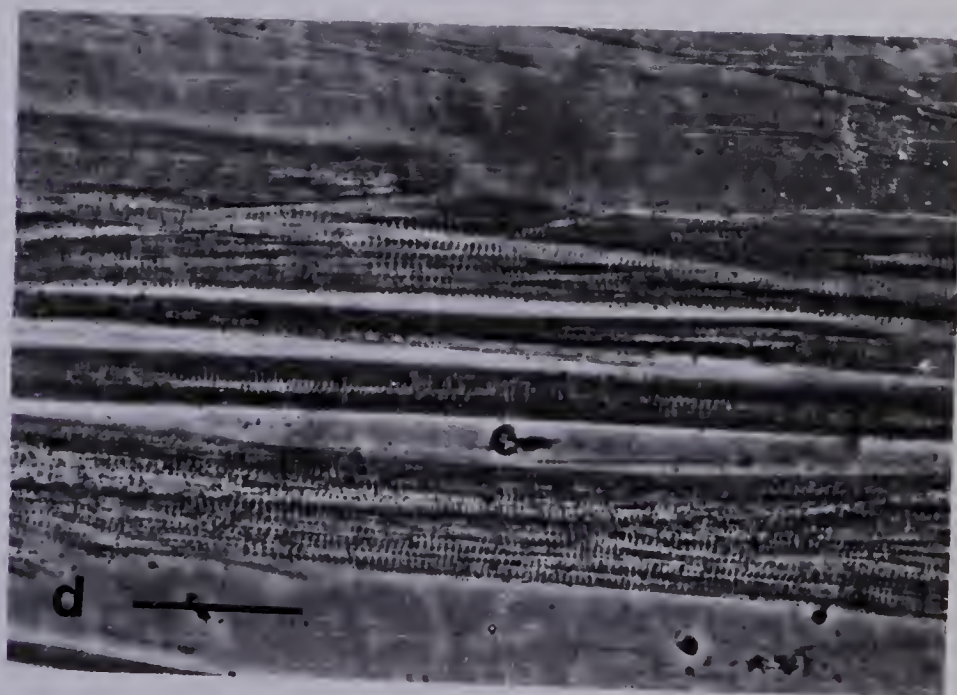
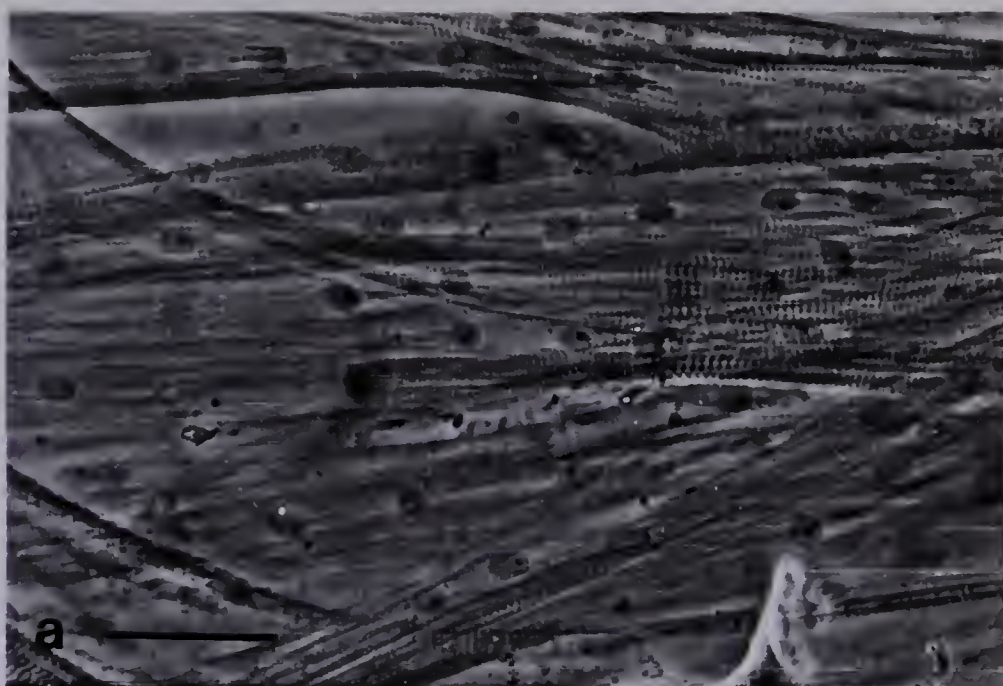
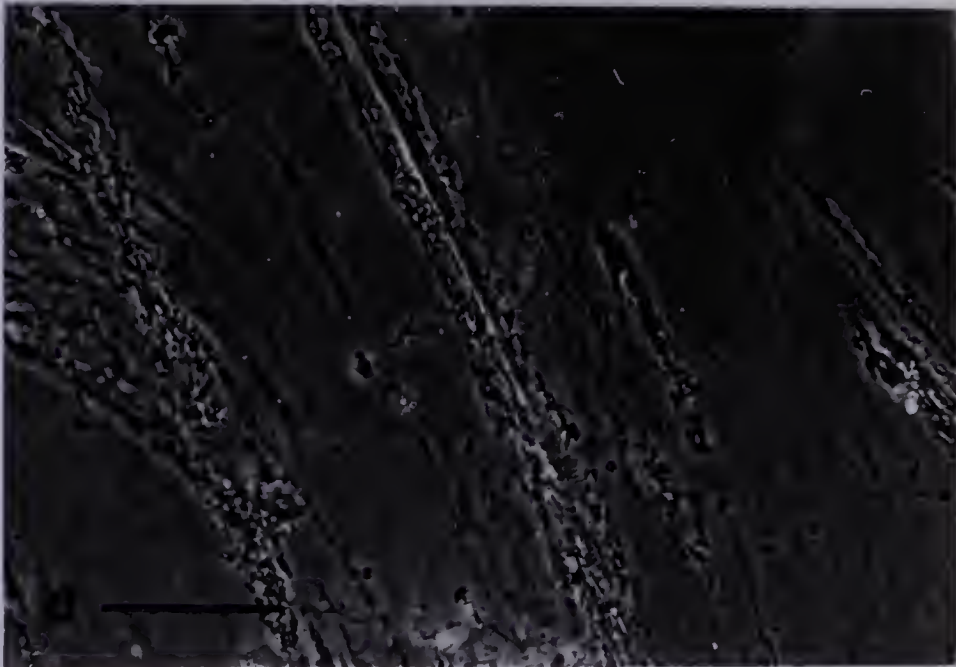
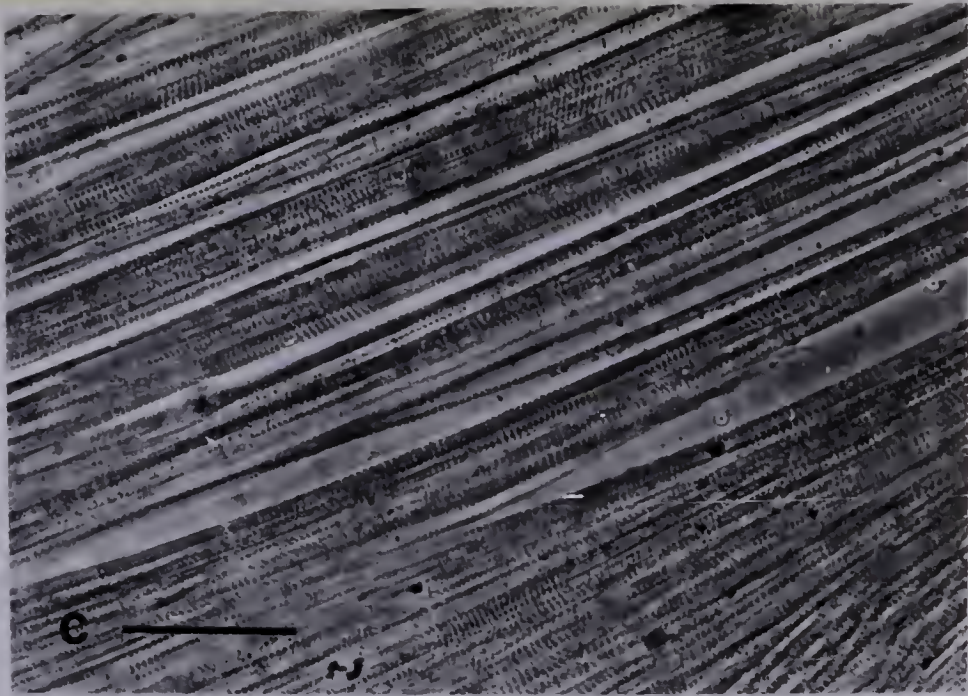


Figure 7. Cytotoxic effect of combined use of protease inhibitors on cultures of dystrophic chick embryo leg muscle. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 7 days with changes of media on days 4 and 6. Treatment with leupeptin (50 ug/ml) plus pepstatin (50 ug/ml) was initiated on day 7 and maintained over the following 72 hours. Cultures were removed from the incubator at 48 and 72 hours rinsed once with RIB representative fields selected and photographed. a) 48 hour control, b) 48 hour, leupeptin and pepstatin, c) 72 hour control, d) 72 hour, leupeptin + pepstatin.





cellular structure suggesting an extensive solubilization of culture protein. Secondly, in some preliminary experiments, a comparison of levels of extractable protein/plate, obtained with either SNU or 0.1 N NaOH/0.4% deoxycholate (Poole and Wibo, 1973) indicated that the SNU buffer was considerably superior. Thus the average amount of protein obtained from five plates (60 mm) of 6 day old normal cultures extracted with SNU was 3.27 ± 0.17 mg whilst with 0.1 N NaOH/0.4% deoxycholate the yield was only 1.37 ± 0.10 mg. (N.B. protein concentrations of the 0.1 N NaOH/0.4% deoxycholate extracts were determined by the Bradford assay as described in Materials and Methods except that the BSA standards were prepared in a one-tenth dilution of 0.1 N NaOH/0.4% deoxycholate.) These results suggest that SNU is a relatively efficient buffer for extraction of protein from the cultures.

The effects of the inhibitors on protein degradation in the cultures were investigated by an isotope decay method as described in Materials and Methods. Typical results obtained from a single set of cultures are shown in Figure 8 and data from three such experiments are summarized in Table 4. Under standard labelling conditions normal cultures incorporated slightly more than 1% of the administered radioactivity into the TCA precipitates of the cell extracts (mean \pm S.E. of three separate experiments was $1.21 \pm 0.13\%$). As can be seen from Figure 8, the semi-logarithmic plots for normal control cultures indicated a complex non-linear pattern of decay suggesting that protein degradation in these cultures did not conform to simple first order kinetics. The rate of loss of

TABLE 4

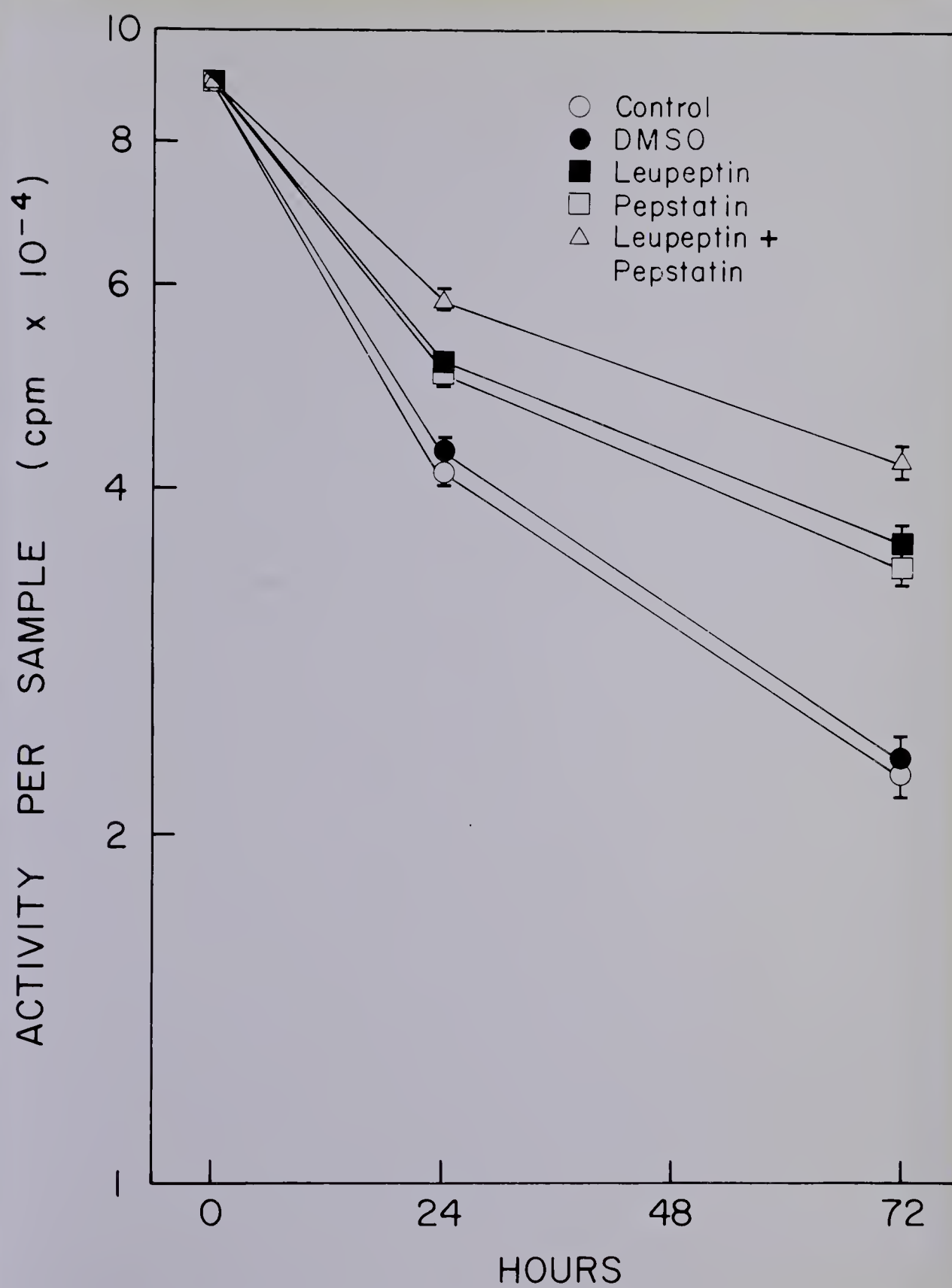
SUMMARY OF EFFECTS OF PROTEASE INHIBITORS ON PROTEIN DEGRADATION IN
NORMAL CHICK EMBRYO CULTURES

	% INHIBITION		AVERAGE DECAY RATES (CPM/hour)	
	<u>24 hr</u>	<u>72 hr</u>	<u>0-24 hr</u>	<u>24-72 hr</u>
CONTROL			2085 \pm 127	185 \pm 10
DMSO			2020 \pm 122 ^d	179 \pm 8 ^d
LEUPEPTIN (L)	18.7 \pm 1.5	19.3 \pm 1.9	1695 \pm 111 ^b	143 \pm 8 ^b
PEPSTATIN (P)	15.9 \pm 1.4	16.9 \pm 1.6	1754 \pm 107 ^b	144 \pm 19 ^d
P & L	33.4 \pm 2.1 ^a	29.9 \pm 2.0 ^a	1390 \pm 49 ^c	166 \pm 58 ^d

Cultures (1×10^6 cells/60 mm plate) were grown for 4 days and labelled with ^{35}S -methionine (3.6 μCi /60 mm plate) between the 4th and 5th days. Addition of inhibitors was initiated at the end of the labelling period (0 hours). Samples were taken at 0, 24 and 72 hours post-labelling, analyzed for TCA precipitable radioactivity and percent inhibition and decay rates determined as described in Materials and Methods. Values are mean \pm S.E. of 3 separate experiments.

- a) significantly different from leupeptin or pepstatin treatments at $p < 0.001$
- b) significantly different from control at $p < 0.05$
- c) significantly different from control at $p < 0.01$
- d) not significantly different from control value in same column $p < 0.05$

Figure 8. Decay curves showing the effect of the microbial protease inhibitors on the loss of radioactivity from TCA precipitates of extractable protein from cultures of normal chick embryo leg muscle. Cultures (1×10^6 cells/60 mm plate) were grown for 4 days and labelled with ^{35}S -methionine (3.6 μCi /60 mm plate) between the 4th and 5th day. The end of the labelling period corresponds to 0 hours in the figure. Addition of leupeptin (50 $\mu\text{g/ml}$), pepstatin (50 $\mu\text{g/ml}$) and DMSO (0.5%) was initiated at 0 hours post-labelling. Sample cultures were taken at 0, 24, and 72 hours post-labelling, extracted with SNU and counts were determined in the TCA precipitable fraction of the extracts, as described in Materials and Methods. Values shown are the total TCA precipitable radioactivity per sample and are the mean \pm S.E. ($n=3$ or 4). Using the Students-t-test inhibitor treatments at 24 and 72 hours were shown to be significantly different from the controls and DMSO treatments of the corresponding time point ($p < 0.01$). Controls and DMSO treatments were not significantly different at either times.



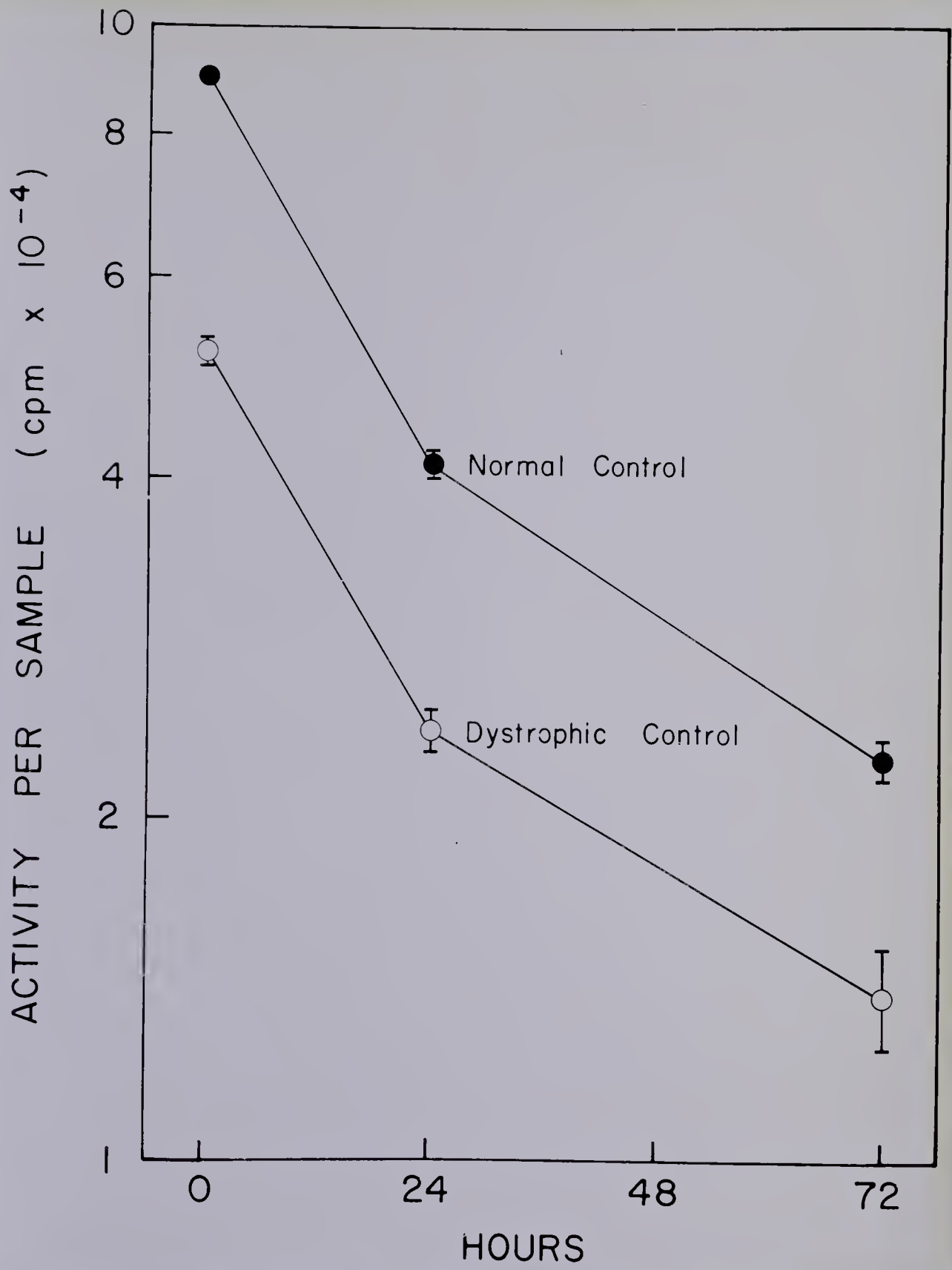
radioactivity from the TCA precipitable fraction of normal cultures decreased from 2085 cpm/hour during the first 24 hours post-labelling to 185 cpm/hour during the 24-72 hour post-labelling interval (Table 4). Almost identical results were obtained from cultures grown in the presence of 0.5% DMSO. (DMSO was used in these experiments to facilitate the uptake of inhibitors in treated cultures (McGowan et al., 1976). The decay curves for DMSO treated were very similar to the controls as is seen in the example shown in Figure 8. The corresponding rates of decay for these curves were 2020 cpm/hour, and 179 cpm/hour in the 0-24 hour and 24-72 hour post-labelling intervals respectively (Table 4). These results suggested that DMSO at the concentration used, did not significantly alter protein degradation in the cultures.

Treatment of the cultures with either leupeptin (50 ug/ml) or pepstatin (50 ug/ml) resulted in a significant decrease with respect to controls in the extent of loss of radioactivity from the TCA precipitable fractions at both 24 and 72 hours post-labelling (Figure 8). The degree of inhibition by leupeptin at 24 and 72 hours post-labelling was 18.7% and 19.3% respectively, while the corresponding values for pepstatin were 15.9% and 16.9% (Table 4). Although it appeared that leupeptin was consistently more effective the difference in degree of inhibition produced was not significantly different from that produced by pepstatin. The effects of the inhibitors were also detectable as significant decreases, with respect to controls in the rates of loss of radioactivity over the 0-24 hour post-labelling interval (Table 4). By contrast only the rates of

radioactivity from the leupeptin samples during the 24-72 hour interval were significantly different from controls (Table 4) suggesting that control rates of degradation had been established in pepstatin treated cultures during this interval. The presence of the inhibitors did not alter the fact that loss of radioactivity occurred in a complex non-linear manner. When the inhibitors were used in combination, (50 ug/ml each) a further decrease with respect to single inhibitor treatments, in the extent of loss of radioactivity at both 24 and 72 hours post-labelling was observed. Thus the inhibition at 24 hours post-labelling increased to 33.4% and to 29.9% at 72 hours post-labelling (Table 4). In addition a significant decrease with respect to controls, in the rate of loss of radioactivity during the 0-24 post-labelling interval was noted in the presence of both inhibitors (Table 4). However, as observed for the pepstatin treatment, the rate of loss of radioactivity in the presence of both inhibitors during the 24-72 hour post-labelling interval was not significantly different from controls again suggesting that establishment of control rates of degradation in these cultures during this interval.

Parallel studies were conducted on cultures derived from genetically dystrophic embryos. Compared to normal cultures of the same age, dystrophic cultures, under standard labelling conditions, incorporated approximately half as much of the administered radioactivity into TCA precipitate of the cell extracts. (See 0 hour values in Figure 9). Thus the mean value of percent incorporation obtained from three separate experiments was $0.52 \pm 0.04\%$. The

Figure 9. Loss of radioactivity from TCA precipitates of extractable cell protein from cultures of both normal and dystrophic chick embryo leg muscle. Decay curves shown are the respective controls from figures 8 and 10 and were determined as described in the legends to those figures.



semi-logarithmic plots of loss of TCA precipitable radioactivity from extracts of dystrophic controls revealed a complex non-linear pattern of decay similar to that seen for normal controls (Figure 9). However the rates of loss of TCA precipitable radioactivity during the 0-24 hour post-labelling interval were significantly lower ($p < 0.01$) in dystrophic controls than in normal controls (Tables 4 and 5), suggesting that protein degradation was slower in dystrophic control cultures at this stage. Conversely the rate of decay during the 24-72 hour interval was higher ($p < 0.05$) in these cultures. Dystrophic cultures grown in the presence of 0.5% DMSO did not differ from dystrophic controls, suggesting that DMSO did not alter protein degradation in these cultures (Table 5, Figure 10).

The presence of leupeptin (50 ug/ml) or pepstatin (50 ug/ml) in the dystrophic cultures resulted in a decrease with respect to the dystrophic controls, in the extent of loss of radioactivity from the TCA precipitable fraction at both 24 and 72 hours post-labelling, indicating that protein degradation had been inhibited in these cultures (Table 5, Figure 10). As was found in the normal cultures, leupeptin appeared to be slightly more effective than pepstatin, but again the difference was not significant. The degree of inhibition produced by either inhibitor at 24 hours post-labelling was similar in both normal and dystrophic cultures. However, at 72 hours post-labelling the inhibition produced in dystrophic cultures was significantly lower ($p < 0.01$) than that observed in normal cultures. With respect to the rates of loss of

TABLE 5

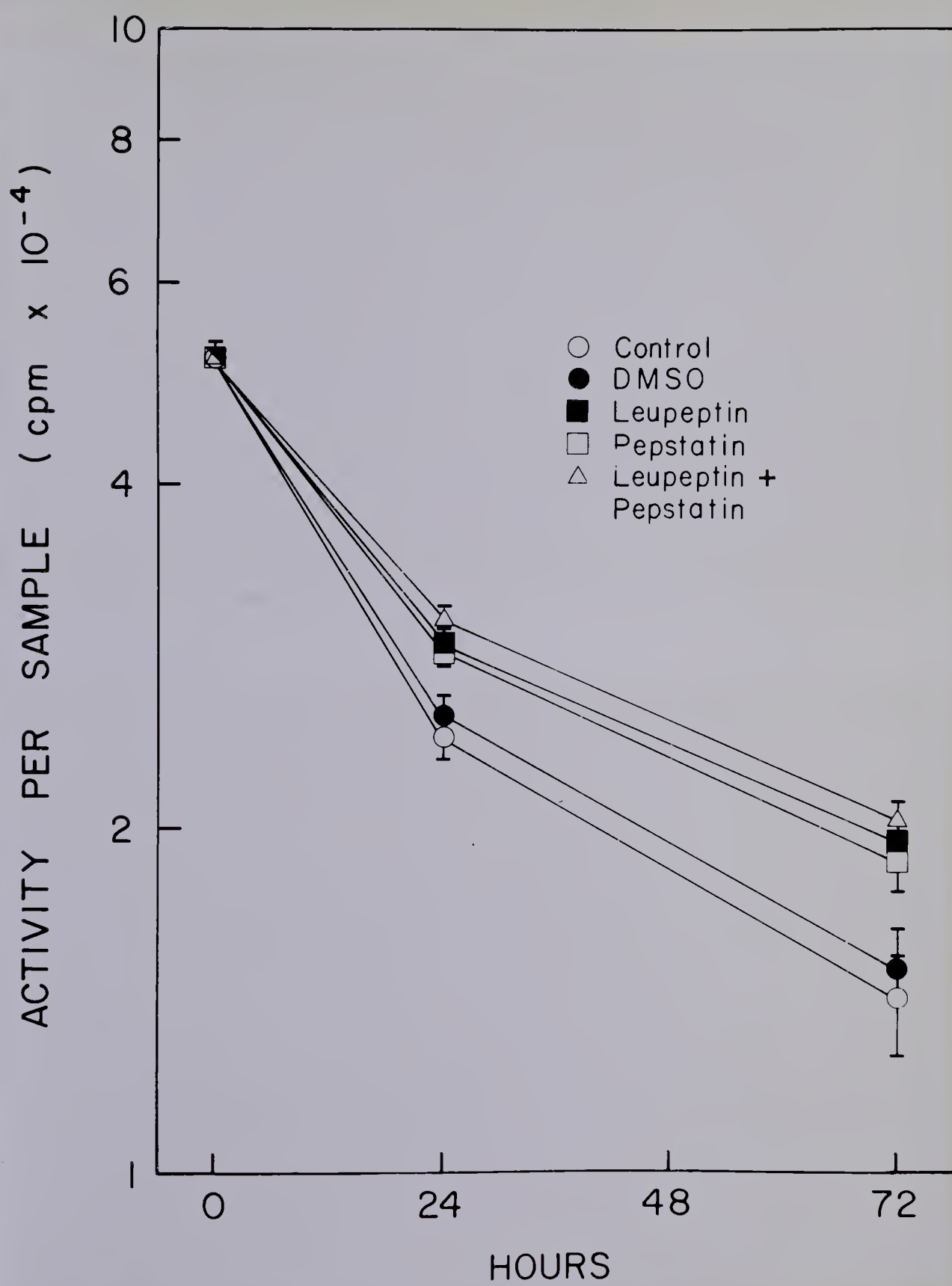
SUMMARY OF EFFECTS OF PROTEASE INHIBITORS ON PROTEIN DEGRADATION IN
DYSTROPHIC CHICK EMBRYO CULTURES

	% INHIBITION		AVERAGE DECAY RATES (CPM/hour)	
	<u>24 hr</u>	<u>72 hr</u>	<u>0-24 hr</u>	<u>24-72 hr</u>
CONTROL			1253 \pm 106	252 \pm 22
DMSO			1219 \pm 134 ^c	244 \pm 15 ^c
LEUPEPTIN (L)	16.1 \pm 1.6	12.1 \pm 1.6	1052 \pm 100 ^b	247 \pm 25 ^c
PEPSTATIN (P)	14.0 \pm 1.9	10.0 \pm 1.4	1078 \pm 98 ^b	252 \pm 35 ^c
P & L	21.6 \pm 1.6 ^a	13.7 \pm 1.7	981 \pm 71 ^b	259 \pm 32 ^c

Cultures (1×10^6 cells/60 mm plate) were grown for 4 days and labelled with ^{35}S -methionine (3.6 μCi /60 mm plate) between the 4th and 5th days. Addition of inhibitors (50 $\mu\text{g/ml}$) and DMSO (0.5%) was initiated at the end of the labelling period (0 hours). Samples were taken at 0, 24 and 72 hours post-labelling, analyzed for TCA precipitable radioactivity, and percent inhibition and average decay rates determined as described in Materials and Methods. Values are mean \pm S.E. of 3 separate experiments.

- a) significantly different from leupeptin or pepstatin treatment at $p < 0.01$
- b) significantly different from control at $p < 0.05$
- c) not significantly different from control value in same column $p < 0.05$.

Figure 10. Decay curves showing the effect of the microbial protease inhibitors on the loss of radioactivity from TCA precipitates of extractable protein from cultures of dystrophic chick embryo leg muscle. Cultures (1×10^6 cells/60mm plate) were grown for 4 days and labelled with ^{35}S -methionine (3.6 $\mu\text{Ci}/60 \text{ mm plate}$) between the 4th and 5th days. The end of the labelling period corresponds to 0 hours in the figure. Addition of leupeptin (50 $\mu\text{g}/1\text{m}$), pepstatin (50 $\mu\text{g}/\text{ml}$), and DMSO (0.5%) was initiated at 0 hours post-labelling. Sample cultures were taken at 0, 24 and 72 hours post-labelling, extracted with SNU and counts were determined in the TCA precipitable fraction of the extracts, as described in Materials and Methods. Values shown are the total TCA precipitable radioactivity per sample and are the mean \pm S.E. ($n=3$ or 4). Using the Students-t-test inhibitor treatments at 24 and 72 hours were shown to be significantly different from the controls and DMSO treatments of the corresponding time point ($p < 0.01$). Controls and DMSO treatments were not significantly different at either time.



radioactivity, there was a significant inhibition with respect to the dystrophic controls, in the rate of loss of radioactivity between 0-24 hours post-labelling, but not during the 24-72 hour post-labelling interval (Table 5). Treatment of dystrophic cultures with both inhibitors simultaneously (50 ug/ml each), resulted in an increase in the degree of inhibition at 24 and 72 hour post-labelling (Table 5) but this increase was considerably smaller than that produced by both inhibitors in the normal cultures (Table 4). The combined use of the inhibitors also produced a significant decrease in the rate of loss of radioactivity between 0-24 hours post-labelling, but not between 24-72 hours post-labelling. In this respect, the combined use of the inhibitors did not differ from that found when each inhibitor was used on its own.

In order to determine if the inhibitors had any effect on the amount of protein in the cultures and if normal and dystrophic cultures differed in this respect, levels of extractable protein were assayed. Table 6 shows data obtained from three separate experiments on normal cultures. During the first 24 hour post-labelling, the level of extractable protein increased in control and treated cultures by similar amounts, indicating that the inhibitors did not influence protein accumulation. The increase was followed by a decline back to approximately the 0 hour levels, and again no significant differences between controls and treatments were found. Corresponding data for dystrophic cultures are shown in Table 7. As can be seen the level of extractable protein at 0 ($p < 0.01$) and 24 hours ($p < 0.05$) post-labelling was significantly less than normal

TABLE 6

EFFECTS OF PROTEASE INHIBITORS ON YIELDS OF EXTRACTABLE CELL PROTEIN
(mg/60mm plate) IN NORMAL CHICK EMBRYO CULTURES

	0 hours	24 hours	72 hours
CONTROL	2.54 \pm 0.13 ^a	3.18 \pm 0.13	2.51 \pm 0.11 ^a
DMSO		3.09 \pm 0.14 ^b	2.55 \pm 0.14 ^b
LEUPEPTIN (L)		3.06 \pm 0.10 ^b	2.39 \pm 0.09 ^b
PEPSTATIN (P)		3.23 \pm 0.11 ^b	2.60 \pm 0.12 ^b
P & L		3.24 \pm 0.12 ^b	2.48 \pm 0.13 ^b

Cultures (1×10^6 cells/60 mm plate) were grown for 5 days with one change of medium on day 4. Addition of inhibitors (50 ug/ml) and DMSO (0.5%) was initiated on day 5 (0 hours) and maintained through day 8 (72 hours). Samples were taken at 0, 24 and 72 hours after addition of inhibitors, extracted with SNU (2ml) and analyzed for protein content as described in Materials and Methods. Values are mean \pm S.E. of three separate experiments.

- a) significantly different from 24 hour control at $p < 0.05$.
- b) not significantly different from control value in same column $p < 0.05$.

TABLE 7

EFFECTS OF PROTEASE INHIBITORS ON YIELDS OF EXTRACTABLE CELL PROTEIN
(mg/60mm plate) IN DYSTROPHIC CHICK EMBRYO CULTURES

	0 hours	24 hours	72 hours
CONTROL	1.42 \pm 0.09 ^a	2.33 \pm 0.08	2.39 \pm 0.07
DMSO		2.28 \pm 0.06 ^b	2.41 \pm 0.11 ^b
LEUPEPTIN (L)		2.23 \pm 0.13 ^b	2.46 \pm 0.10 ^b
PEPSTATIN (P)		2.36 \pm 0.14 ^b	2.34 \pm 0.14 ^b
P & L		2.27 \pm 0.09 ^b	2.29 \pm 0.09 ^b

Cultures (1×10^6 cells/60mm plate) were grown for 5 days with one change of medium on day 4. Addition of inhibitors (50 ug/ml) and DMSO (0.5%) was initiated on day 5 (0 hours) and maintained through day 8 (72 hours). Samples were taken at 0, 24 and 72 hours after addition of inhibitors, extracted with SNU (2ml) and analyzed for protein content as described in Materials and Methods. Values are mean \pm S.E. of three separate experiments.

- a) significantly different from 24 hour control at $p < 0.01$.
- b) not significantly different from control value in same column $p < 0.05$.

cultures, but similar at 72 hours post-labelling. An increase during the first 24 hours was also seen, but in contrast to the situation in normal cultures the levels of extractable protein at 72 hours post-labelling, were similar to those at 24 hour post-labelling. In addition the inhibitors did not influence either the level of extractable proteins or the pattern of changes during the experimental period. Thus in spite of the fact that the inhibitors appeared to block protein degradation there was no evidence to suggest that this was accompanied by changes in the accumulation of protein in the cultures. One possible explanation for the failure to detect differences in the amount of protein is that protein synthesis may also have been influenced, either directly or indirectly by the inhibitors (Gunn, 1978). Experiments were therefore conducted to determine whether or not the inhibitors influenced protein synthesis in the cultures. In order to do this the extent of incorporation of radioactivity into the TCA precipitable fraction of the cell extracts from cultures pre-incubated with inhibitors (50 ug/ml) was estimated (see Materials and Methods for details). Table 8 shows combined data from three separate experiments. No significant differences in the extent of incorporation were found under any of the conditions tested suggesting that the presence of the inhibitors did not influence protein synthesis during the interval studied. Table 9 shows the combined data from two similar experiments conducted on cultures prepared from dystrophic embryos. A significantly ($p < 0.01$) lower level of

TABLE 8

EFFECT OF PROTEASE INHIBITORS ON INCORPORATION OF ^{35}S -METHIONINE
IN NORMAL CULTURES OF CHICK EMBRYOS

	cpm/sample
CONTROL	28576 \pm 1479
DMSO	27727 \pm 627 NS
LEUPEPTIN (L)	26517 \pm 941 NS
PEPSTATIN (P)	26950 \pm 1250 NS
P + L	27124 \pm 581 NS

Cultures (1×10^6 cells/60 mm plate) were grown for 4 days at which time the media was changed and addition of inhibitors (50 ug/ml) and DMSO (0.5%) initiated. On day 5, the media was changed again and the cells labelled with ^{35}S -methionine for 12 hours. Cultures were then extracted with SNU and analyzed for TCA precipitable radioactivity as described in Materials and Methods. Values are mean \pm S.E. of three separate experiments.

N.S. = not significantly different from control $p < 0.05$.

TABLE 9

EFFECT OF PROTEASE INHIBITORS ON INCORPORATION OF ^{35}S -METHIONINE
IN DYSTROPHIC CHICK EMBRYO CULTURES

	cpm/sample	
CONTROL	12959 \pm 1017	
DMSO	13275 \pm 498	NS
LEUPEPTIN (L)	11892 \pm 668	NS
PEPSTATIN (P)	11308 \pm 908	NS
P + L	13292 \pm 565	NS

Cultures (1×10^6 cells/60 mm plate) were grown for 4 days at which time the media was changed and addition of inhibitors (50 ug/ml) and DMSO (0.5%) initiated. On day 5, the media was changed again and the cells labelled with ^{35}S -methionine for 12 hours. Cultures were then extracted with SNU and analyzed for TCA precipitable radioactivity as described in Materials and Methods. Values are mean \pm S.E. for three separate experiments.

N.S. = not significantly different from control $p < 0.05$.

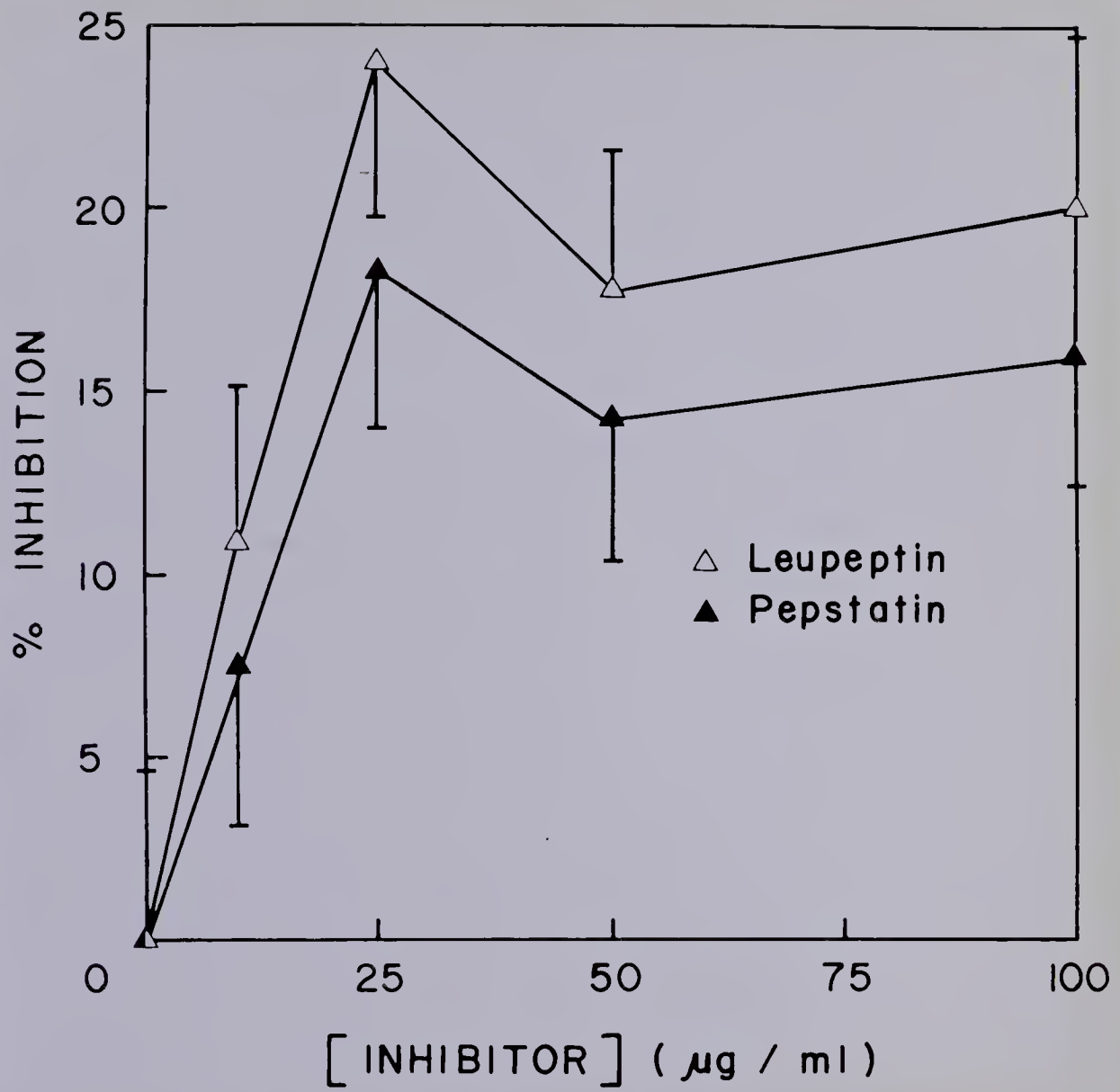
incorporation compared to normal cultures was noted in these experiments. However, similar to normal cultures, no significant differences in the extent of incorporation was found under any of the conditions tested, suggesting that the inhibitors did not effect protein synthesis in these cultures.

Since the combined use of the inhibitors had given rise to increased levels of inhibition in the normal cultures at both 24 and 72 hours post-labelling, and in the dystrophic cultures at 24 hours post-labelling, the relationship between inhibitor concentration and the degree of inhibition produced was investigated (Figure 11). Inhibition, by both leupeptin and pepstatin increased sharply to maximum values of 22.4% and 19.0%, at a concentration of 25 ug/ml. At higher concentrations (50 and 100 ug/ml) there was a slight decline in the level of inhibition to 18.0-20.0% for leupeptin and 15.0-17.0% for pepstatin. However, these levels of inhibition were not significantly different from those obtained at 25 ug/ml. It therefore appeared that a plateau of inhibitory activity was achieved in the 25-100 ug/ml region.

C. Procedure for Isolation of Myofibrils from Primary Cultures of Skeletal Muscle

In the previous section, the effects of the protease inhibitors on several aspects of "extractable protein" metabolism, in normal and dystrophic cultures were described. Since part of the cellular material in these cultures is non-myogenic, the results obtained during these studies must necessarily reflect a contribution from this source, and to some extent obscure the behavior of the specific

Figure 11. Relationship between microbial protease inhibitor concentration and inhibition of protein degradation in cultures of normal chick embryo leg muscle. Cultures (1×10^6 cells/60 mm plate) were grown for 4 days and labelled with ^{35}S -methionine between the 4th and 5th day. Addition of leupeptin, and pepstatin (both in the presence of 0.5% DMSO) at the concentrations indicated in the figure were initiated immediately after labelling. Sample cultures were taken at 0 and 24 hours post-labelling, extracted with SNU and counts determined in the TCA precipitable fraction of the extracts, as described in Materials and Methods. Percentage inhibition was obtained by subtracting control counts at 24 hours from treatment counts at 24 hours and expressing the difference as a percentage of the difference between the 24 hour control and 0 hour, counts. Values are mean \pm S.E. of one experiment. For a given inhibitor, treatment at 10 ug/ml produced significantly lower inhibition than at 25 ug/ml ($p < 0.05$). No other significant differences were noted.



muscle proteins. It was therefore decided to examine more specifically effects of the inhibitors on the muscle tissue proteins by studying the contractile apparatus. As before, normal and dystrophic cultures were also compared in this respect. In order to do this a reliable procedure for the isolation of myofibrils from the cultures was developed. During development of the procedure, only cultures exhibiting clearly cross-striated myotubes and spontaneous contractions (8 or 9 days old), were used, although the method was subsequently tested on less developed cultures.

Initial attempts to isolate myofibrils using published procedures (La Grange and Low, 1976; Bester and Gevers, 1975; Allen et al., 1978) were unsuccessful as judged by phase contrast microscopy and sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In these cases the "myofibrillar" pellet when viewed by phase contrast microscopy, characteristically appeared as large clumps of granular material, lacking recognizable structural features, such as distinct cross-striations (Figure 12). Attempts to analyze such preparations by SDS-PAGE resulted in samples which did not completely dissolve in detergent and gels which exhibited streaking rather than distinct band formation. It was not clear from these results whether the myofibrillar material was being lost during isolation procedures and therefore not present in the final pellet or whether it was present in a grossly distorted unrecognizable form and unresolved from other contaminating



Figure 12. Myofibrillar pellet obtained from cultures of normal chick embryo leg muscle by the method of Allen et al. (1978). Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 8 days with changes of media on days 4 and 6. Myofibrils were then isolated by the method of Allen et al. (1978) and the resulting pellet photographed.

Calibration bar 50 μ m.

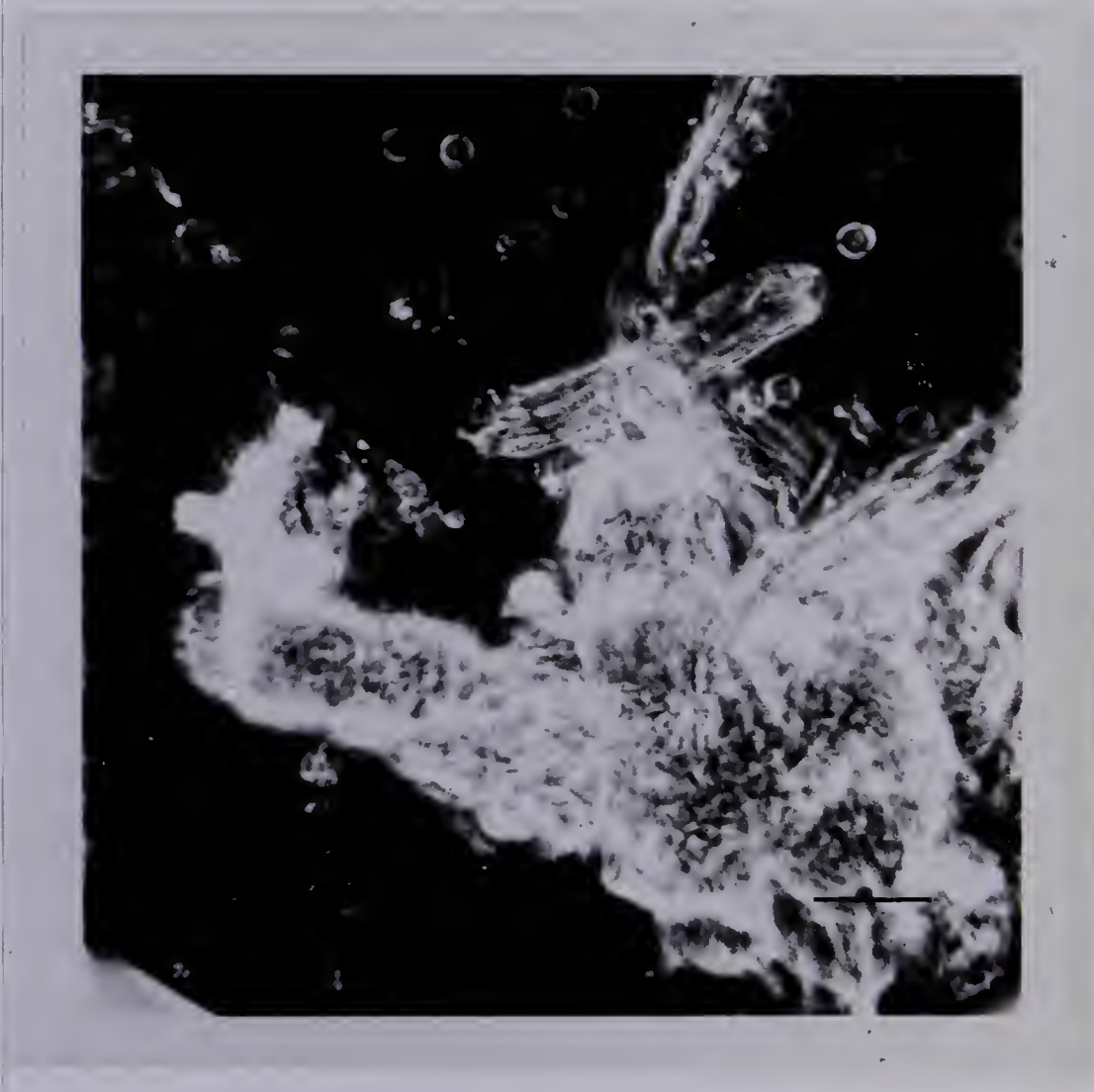
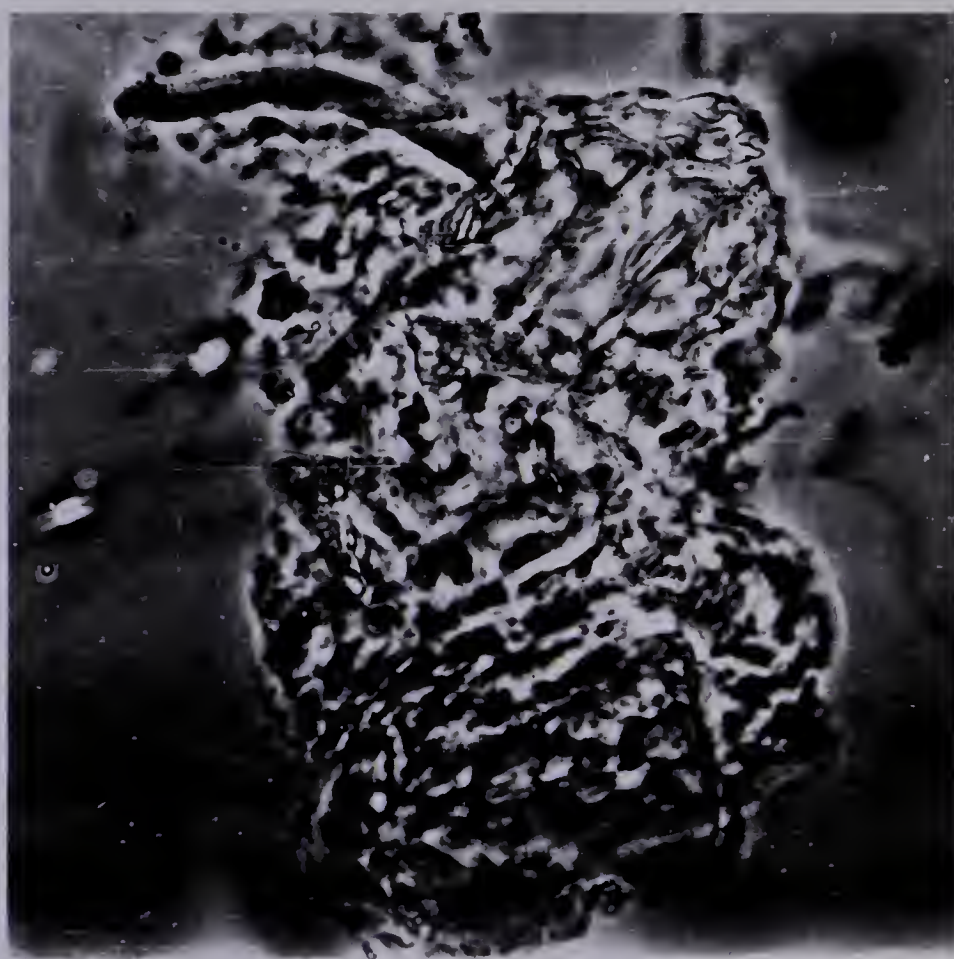


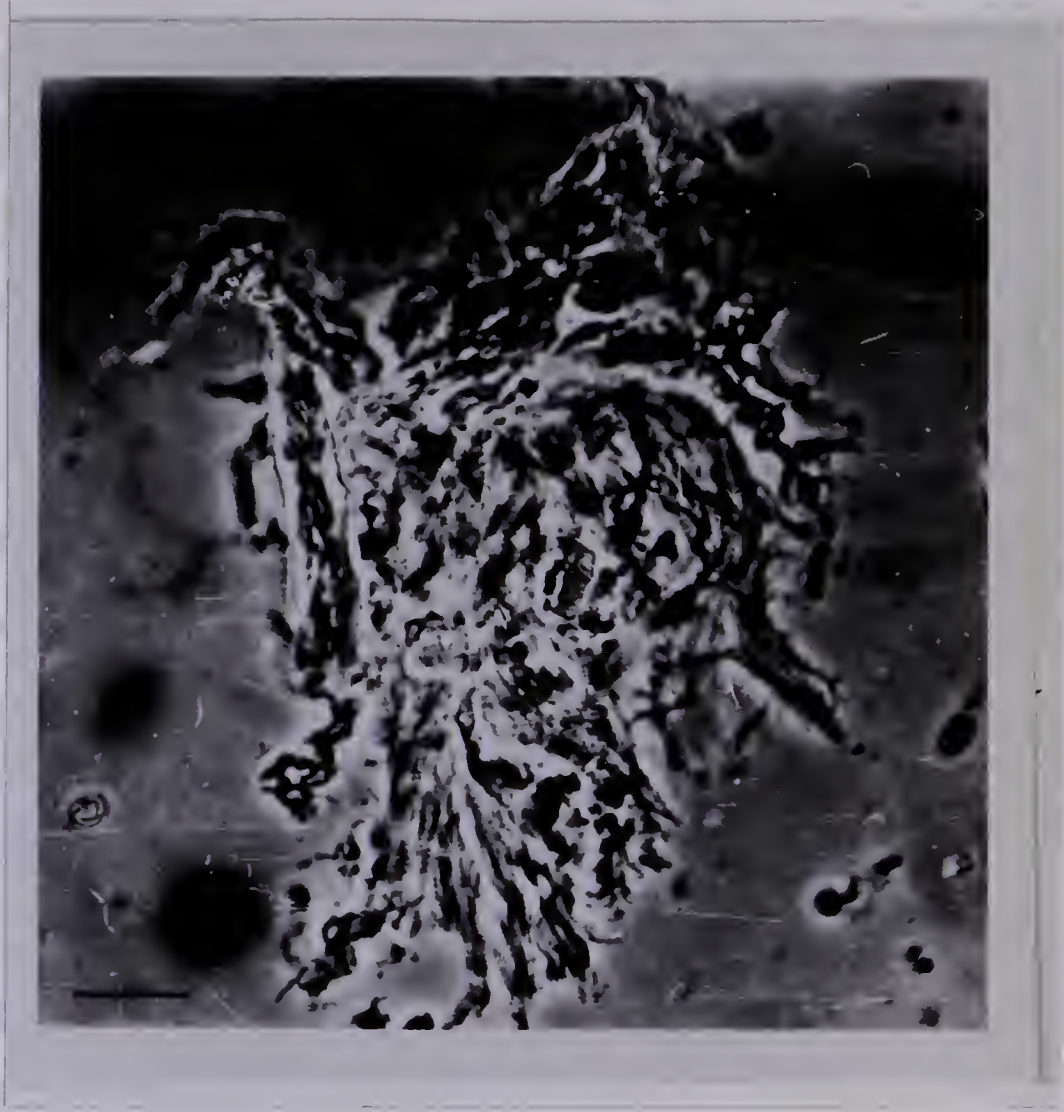
Figure 13. Myofibrillar pellet from cultures of normal chick embryo leg muscle after treatment with crude collagenase. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 8 days with changes of media on days 4 and 6. Myofibrils were then isolated by the method of Allen et al. (1978) treated with crude collagenase (0.01% Hanks balanced salts, 30 min., 25°C) and photographed. Calibration bar 50 μ m



materials, eg. nuclei, connective tissue. The presence of contamination such as nuclei could interfere with the protein/detergent interaction and solubilization (Paterson and Strohman, 1972) and possibly lead to the failure to obtain adequately resolved bands on the gels. In a study of myofibril isolation from small muscles in the hind limb of the rat, Kohn (1968) reported a disorganized granular appearance in his preparation, which he attributed to collagen contamination. Brief incubation with a crude collagenase removed the granularity and resulted in a preparation of clearly cross-striated single myofibrils. Treatment with collagenase for up to 30 minutes, did not significantly alter the appearance of the granular material derived from the cultures (Figure 13), nor did it result in improvements with respect to SDS solubilization or electrophoresis. In view of the ultimate objectives of this study, more vigorous proteolytic digestions, with for example trypsin, were considered unsuitable as means of purification. As already mentioned, contamination by nuclear material could conceivably account for some of the problems encountered. Consequently incubation of the granular pellet with DNAase for periods up to 30 minutes was tested, but as with collagenase, no significant improvements were noted (Figure 14).

In the isolation of myofibrils from adult skeletal muscle Zak et al., (1972) stressed the importance of relaxation of the tissue (a Ca^{++} chelator, usually EGTA was used), prior to disruption. This resulted in a softening of the tissue, allowing for milder homogenization and subsequent easier separation of the

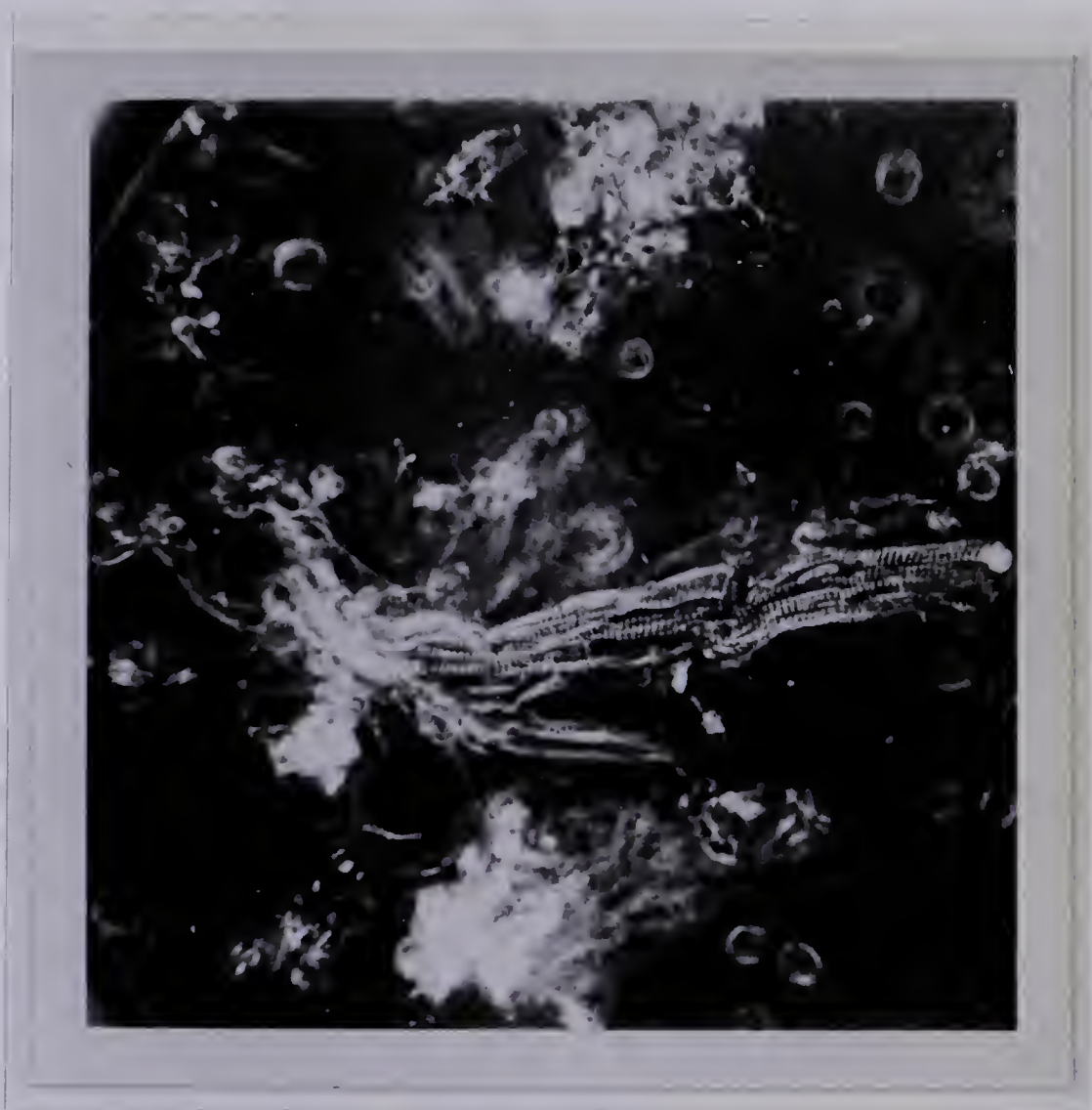
Figure 14. Myofibrillar pellet from cultures of normal chick embryo leg muscle after treatment with DNAase (0.005% in Hanks balanced salt, 25°C). Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 8 days with changes in media on days 4 and 6. Myofibrils were then isolated by the method of Allen et al., (1978) and treated with DNAase. Calibration bar 50 μ m.



myofibrils from contaminants. In some of the quoted procedures (Bester and Gevers, 1975; Allen et al., 1978) similar steps had been included but in contrast to the situation in the adult muscle, did not facilitate isolation of the myofibrils. This seemed to suggest that relaxation of the cultured muscle was not a critical factor in preventing loss of structural features and isolation of myofibrils from cultured muscle. However microscopic examination indicated that loss of the cross-striations, readily visible in the original cultures, occurred at about the same stage as the development of the granular appearance, and that this transformation occurred early in the procedure, possibly during the initial scraping of the cells from the plates. These observations led to the consideration that the cross-striated myotubes, while attached to the culture dish, were prevented from shortening irreversibly, but during scraping, even in the presence of a relaxing medium, the physical restraining force opposing shortening, is removed and the myotubes spontaneously undergo extensive irreversible supercontracture. Such supercontracted cells would be expected to lose their normal structural organization and entrap other materials, precluding the possibility of obtaining a reasonably pure and structurally intact preparation of myofibrils. It was therefore decided to induce prolonged relaxation of the muscle prior to detachment in an attempt to circumvent this occurrence. Accordingly cultures were washed in the relaxing medium (see page 134) for up to 10 minutes prior to detachment and subsequent processing.



Figure 15. Myofibrillar pellet from cultures of normal chick embryo leg muscle treated with relaxing medium. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 8 days with changes of media on days 4 and 6. Medium was then decanted and cultures treated with a relaxing medium (5.0 mM KCl, 2.0 mM EDTA, 10 mM Tris-HCl pH 7.6) for 10 min at -4°C . Myofibrils were then isolated by the method of Allen et al., (1978) and the resulting pellet photographed. Calibration bar 50 μm .



The final "myofibrillar" pellet obtained in these experiments (Figure 15) showed considerable improvement over previous procedures, with respect to microscopic appearance. As can be seen some cross-striated material is present although contamination with granular and other unidentified material is also evident. Attempts to solubilize with detergent and perform electrophoresis with these samples were however still unsuccessful.

In spite of this, the results of these experiments did tend to support the idea that extensive shortening of the myotubes during detachment was a source of difficulty in myofibril isolation. A protocol was therefore devised which attempted to prevent shortening during detachment by fixing the myofibrils in a state of "rigor" (Figure 16). (N.B. In this context, the term "rigor" is not used in the strict sense of the condition occurring in post-mortem muscle, but is simply meant to imply a state in which shortening is prevented.)

Cross-striated myotubes, while still attached to the plate (substratum) were exposed to a low potassium (5.0 mM KCl) buffer containing the calcium chelator ethylenediamine tetracetic acid (EDTA 2.0 mM). Within about one minute of exposure to this buffer, spherical swellings, which increased in size with time, appeared along the surface of the myotubes, (Figure 17a). The nature of these swellings was not established but they are strikingly similar to a phenomenon known as blebbing or potocytosis (Zollinger, 1948), which is apparently related to fluctuating divalent cation concen-

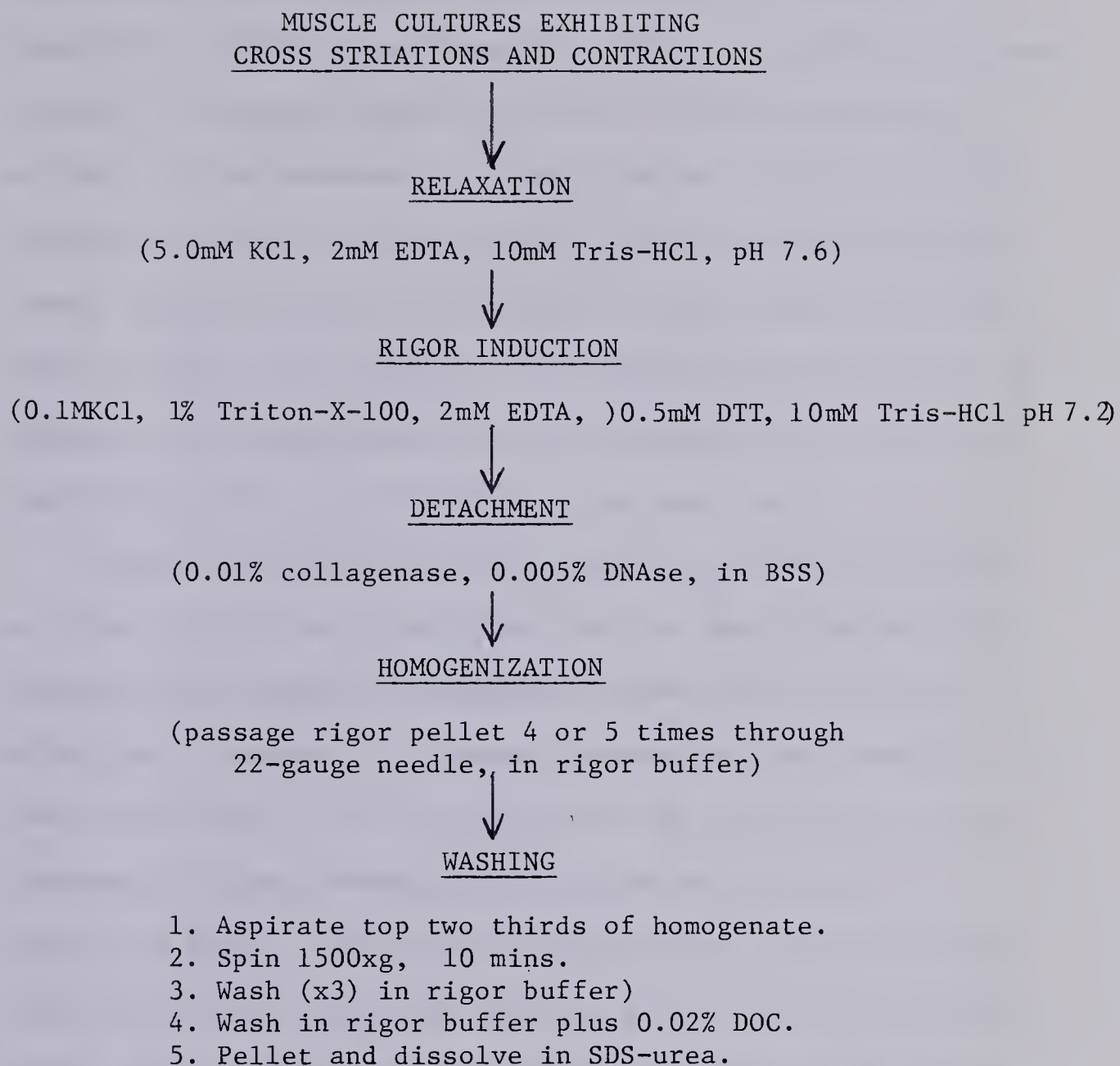


Fig. 16. Flow diagram of the principle steps in myofibril isolation procedure.

trations in the cytoplasm resulting from loss of membrane selectivity (Dornfeld and Owczarzak, 1958). In addition to these changes the myotubes became less clearly striated and had a slightly grainy appearance. It was also noted that many of the mononucleated cells which occupied interstices between the myotubes, became spherical and detached. Gentle agitation of the plate at this stage assisted in dislodging the mononucleated cells. However, prolonged exposure to the relaxing buffer coupled with agitation resulted in detachment of myotubes also.

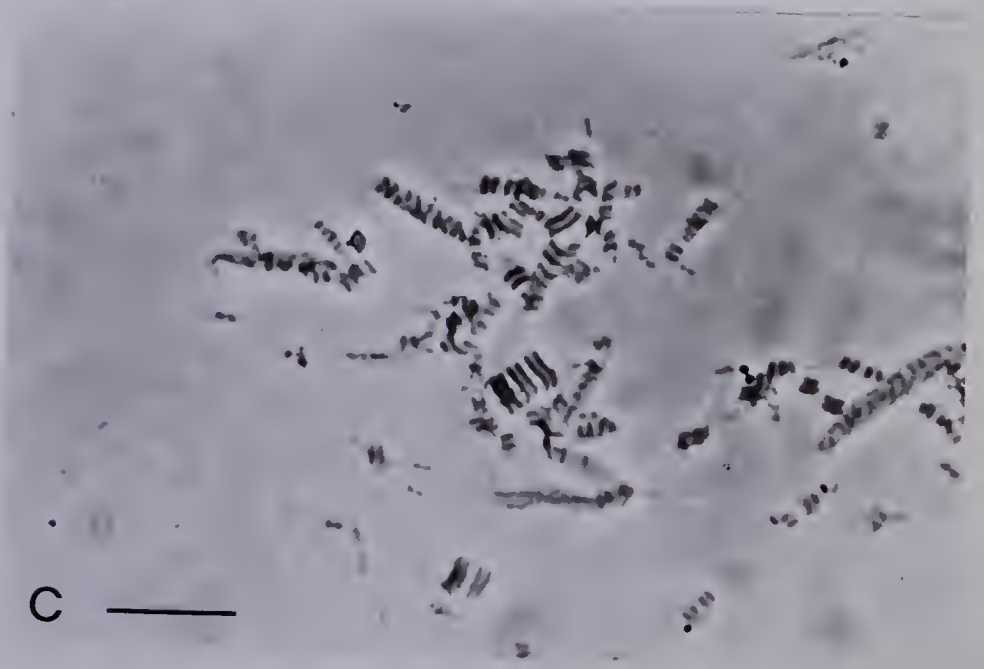
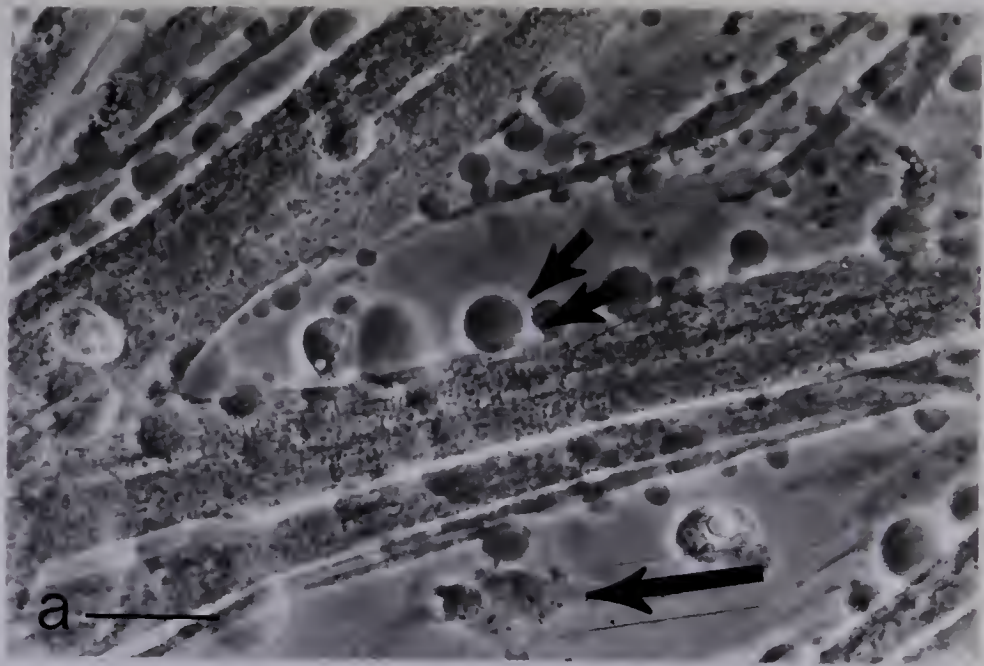
Several possibilities were considered as means of inducing a "rigor" state, but it was found that the use of buffer (RIB) containing the non-ionic detergent Triton-X-100 (2%) was most effective. Treatment of attached myotubes with RIB resulted in a very rapid loss of the bleb structures and graininess, and the appearance of very distinct cross-striations and chains of associated nuclei. The cross-striations were evident throughout the length of the individual myotubes and were clearly exhibited by the vast majority of myotubes in the culture. During the relaxation phase some of the myotubes had become detached at one end and on addition of the RIB these cells could be seen to change from an apparently twisted and flexible state to a very straight and rigid one. Somewhat surprisingly there was no indication that detergent treatment caused any loosening or detachment of the myotubes. The overall impression from microscopic examination at this stage was that the "rigor" state had been successfully induced

Figure 17. Stages in the preparation of myofibrils by standard procedures from cultures of normal chick leg muscle. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 8 days with changes of media on days 4 and 6. Myofibrils were then prepared as described in Materials and Methods.

a) 8 day-old culture of normal chick embryo leg muscle treated with relaxing medium (5.0 mM KCl, 2.0 mM EDTA, 10 mM Tris-HCl pH 7.6) for 2 minutes at 25°C. Double arrow indicates "blebbing" along myotube surface. Single arrow indicates fibroblast. Calibration bar 50 μ m.

b) Same field as above treated with RIB (2% Triton X-100, 2.0 mM EDTA, 0.1 M KCl, 0.5 mM DTT, 10 mM Tris-HCl pH 7.2) 1 minute at 0°C. Arrow indicates region of cross striations. Calibration bar 50 μ m.

c) Myofibrillar pellet obtained from above cultures after detachment with collagenase-DNAase and homogenization. NB, the short myofibrillar fragments and single sarcomeres. Calibration bar 10 μ m.



without serious disruption of the general intracellular organization and particularly the contractile apparatus (Figure 17b).

Attempts to detach the rigor state cells by scraping with a rubber policeman tended to result in clumping of the material and generally a loss of the clearly striated appearance. A milder method of detachment by brief exposure to a collagenase/DNAase mixture was therefore tested and found to be a considerable improvement. The detached material after pelleting at low speed was homogenized by several different procedures. Disruption by vortex as used by Allen et al., (1978), resulted in suspensions containing large clusters of myofibrillar material, often associated with nuclei. On the other hand the use of a tight fitting hand held glass homogenizer resulted in almost complete disappearance of striated material from the suspension. The method found to be most successful was to pass the suspension slowly through a cannula (22-gauge, 3") several times. This procedure worked best if the pellet was first resuspended in a relatively large volume of RIB (2.0 ml) using a vortex for a few seconds. If the pellet was resuspended in smaller volumes of RIB eg. 0.5 ml., homogenization resulted in a variable amount of clumping and loss of cross-striations. An example of the final myofibrillar pellet is shown in Figure 17c. The preparation is essentially free of nuclear contamination and is composed of short strands of distinctly cross-striated myofibrils. It was consistently noted that many of the myofibril fragments were either single sarcomeres or only a few sarcomeres in length.

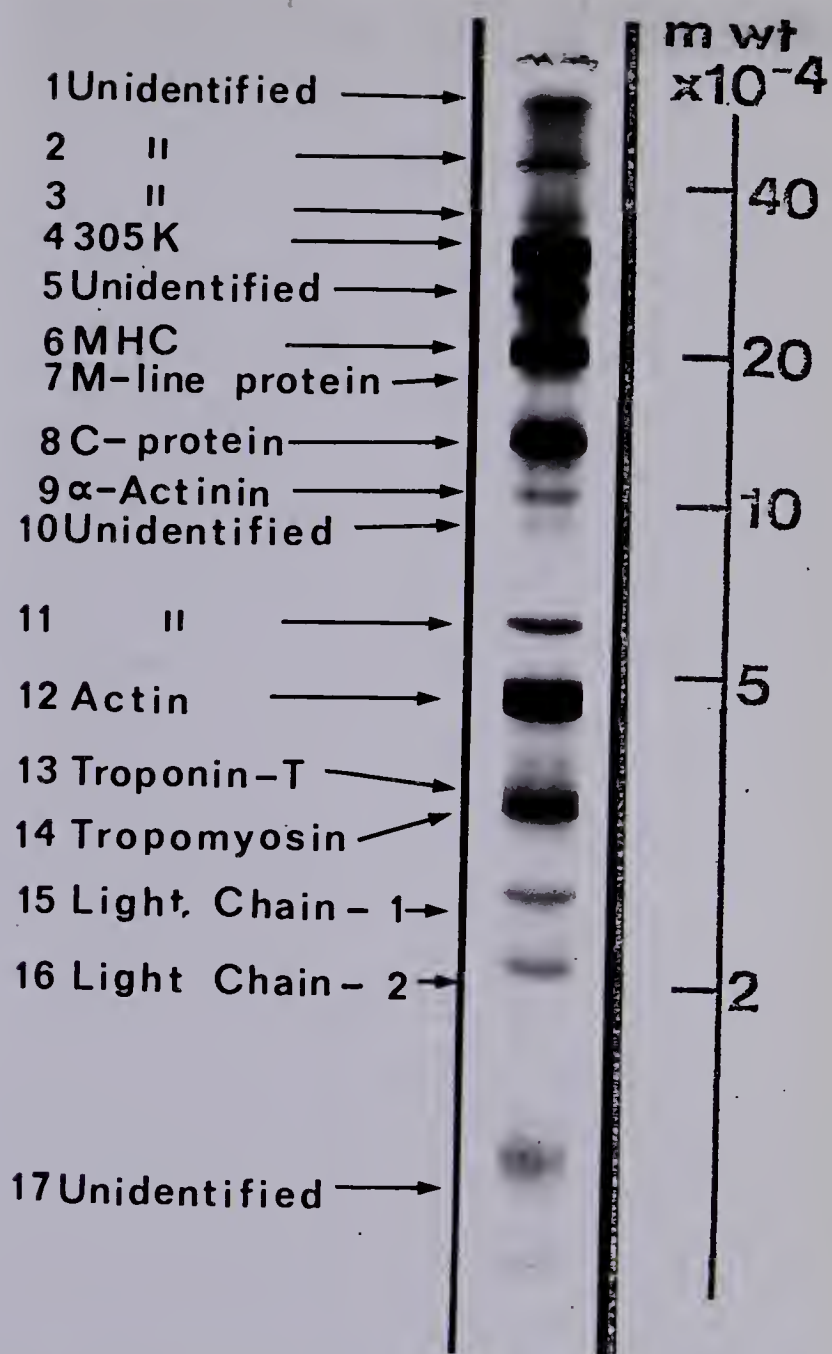
Such samples were found to be readily soluble in SDS and electrophoresis (Figure 18) resulted in gels exhibiting a number of well resolved bands. Both the pattern of banding and the relative distribution of bands was found to be reproducible.

Estimates of molecular weights were obtained by comparison of the relative mobility of the individual components with the calibration curve (Figure 19) constructed from standard proteins of known molecular weights. On the basis of these determinations, tentative identification of several bands was made (Figure 18, Table 10).

A number of unidentified very high molecular weight proteins (bands 1-5) were clearly resolved at the top of the gels. The most prominent band (#4) among this group exhibited a molecular weight of 305,000 daltons, with a second less prominent band (#5) at 255,000 daltons. Running below this was a major component (band 6) at 200,000 daltons which was believed to be myosin heavy chain (MHC). Immediately below MHC was a very faint band (#7) with a molecular weight of 180,000 daltons, which may have corresponded to one of the M-line proteins (Porzio and Pearson, 1977).. Another prominent but diffuse band (#8), possibly C-protein was noted at 140,000 daltons. Two relatively minor bands (#9,10) with molecular weights of 105,000 daltons and 90,000 daltons could also be seen. The heavier of these two bands may have been α -actinin. The region of the gel between these high molecular weight components and actin (band 12, 48,000 daltons) was clear with the exception of a narrow band (#11) at 65,000 which may have corresponded to tropomyosin



Figure 18, SDS-PAGE of standard myofibrillar pellet obtained from cultures of normal chick embryo leg muscle. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 8 days with changes of media on days 4 and 6. Myofibrils were then isolated by the standard procedure and analyzed by SDS-PAGE as described in Materials and Methods.



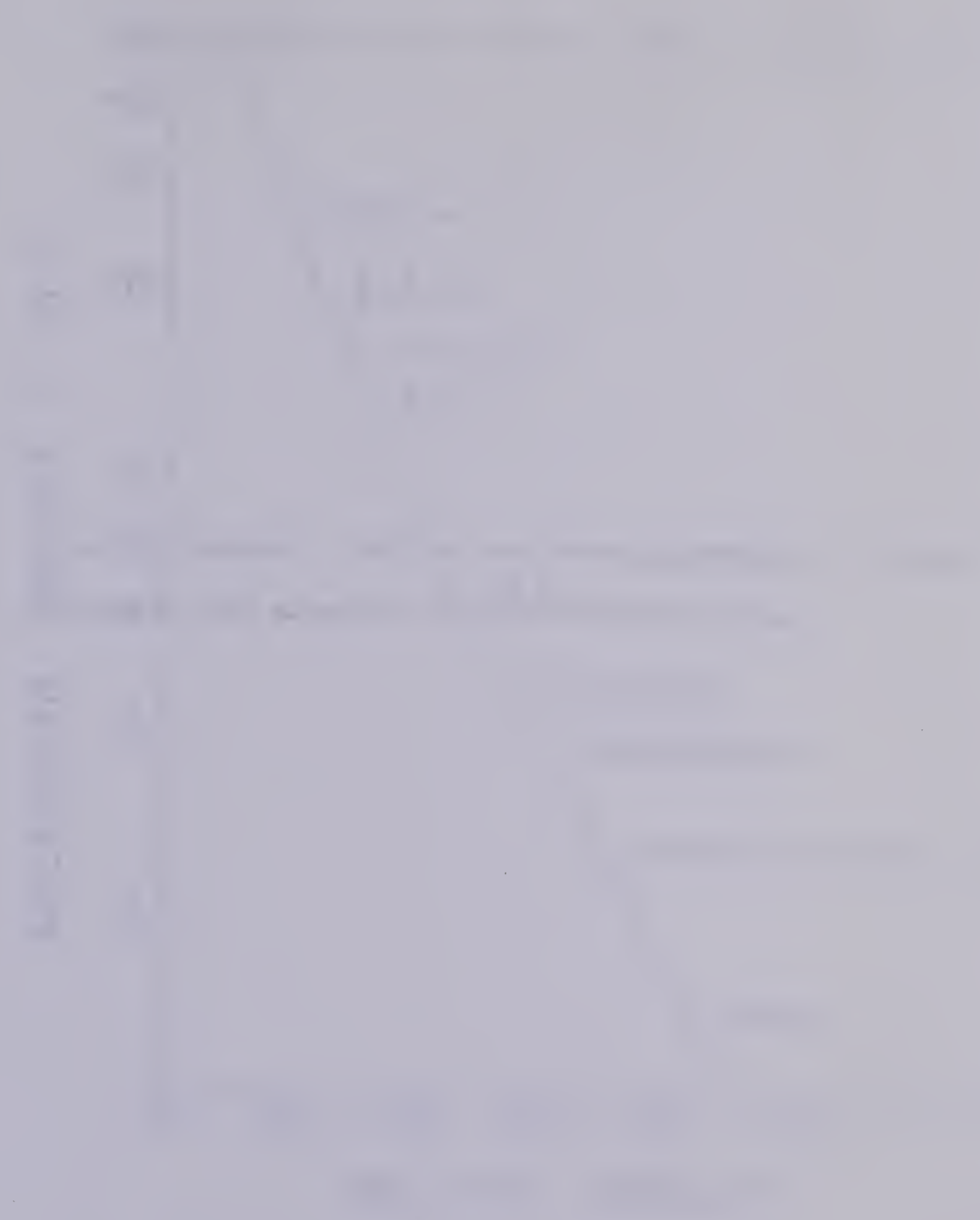


Figure 19. Calibration curve for SDS-PAGE. Standard protein mixture was obtained from Pharmacia Fine Chemicals.

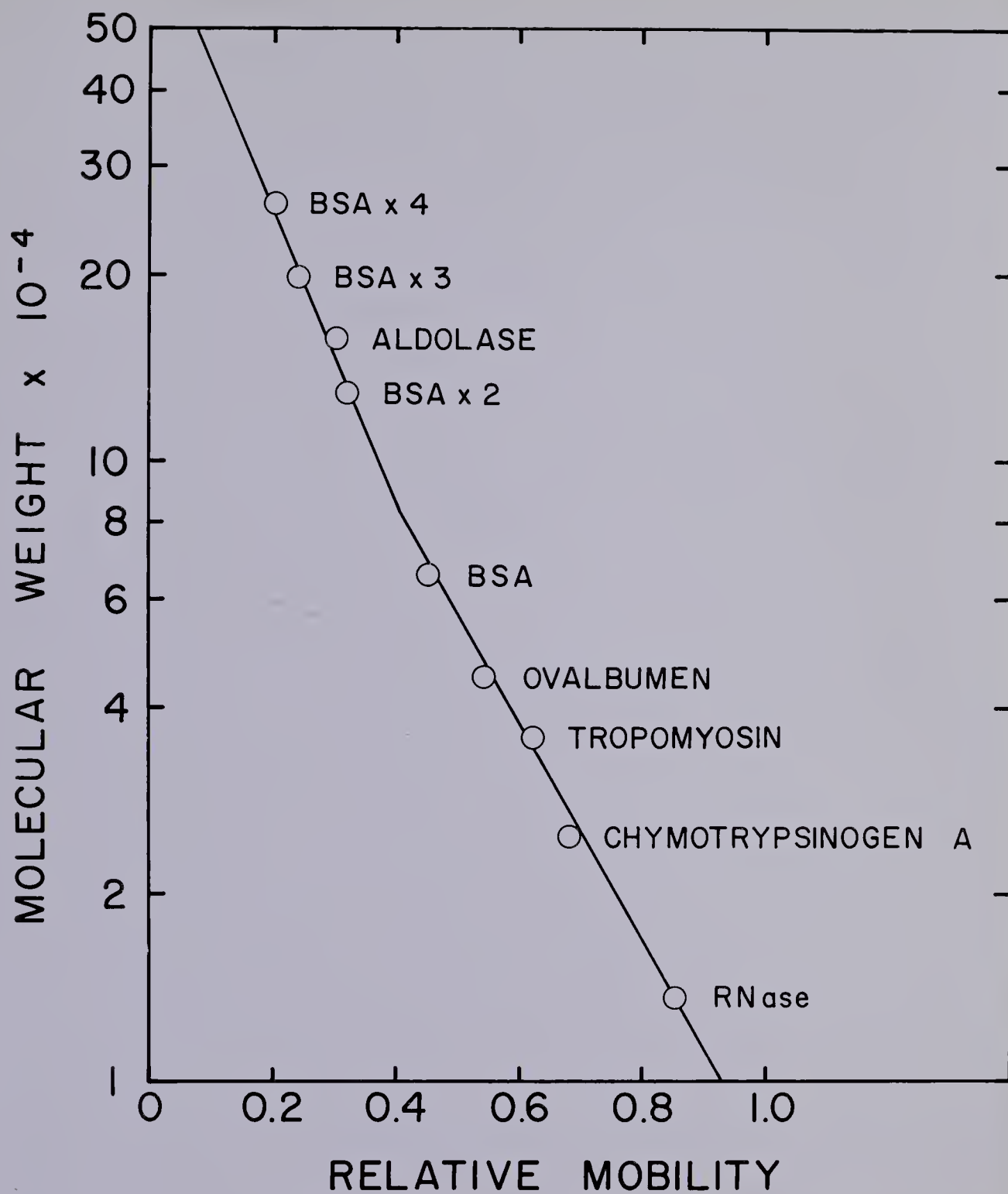


TABLE 10
COMPOSITION OF MYOFIBRILS DERIVED FROM CULTURES OF NORMAL CHICK
EMBRYO SKELETAL MUSCLE

BAND	PROTEIN*	MOLECULAR WT. (Daltons)
1	Unidentified	<500,000
2	"	465,000
3	"	360,000
4	"	305,000
5	"	255,000
6	Myosin Heavy Chain (MHC)	200,000
7	M-Line Protein	180,000
8	C-Protein	140,000
9	α -Actinin	105,000
10	Unidentified	90,000
11	Tropomyosin Dimer	65,000
12	Actin	48,000
13	Troponin-T	37,000
14	Tropomyosin	35,000
15	Light Chain I (LC ₁)	26,000
16	Light Chain II (LC ₂)	20,000
17	Unidentified	12,500

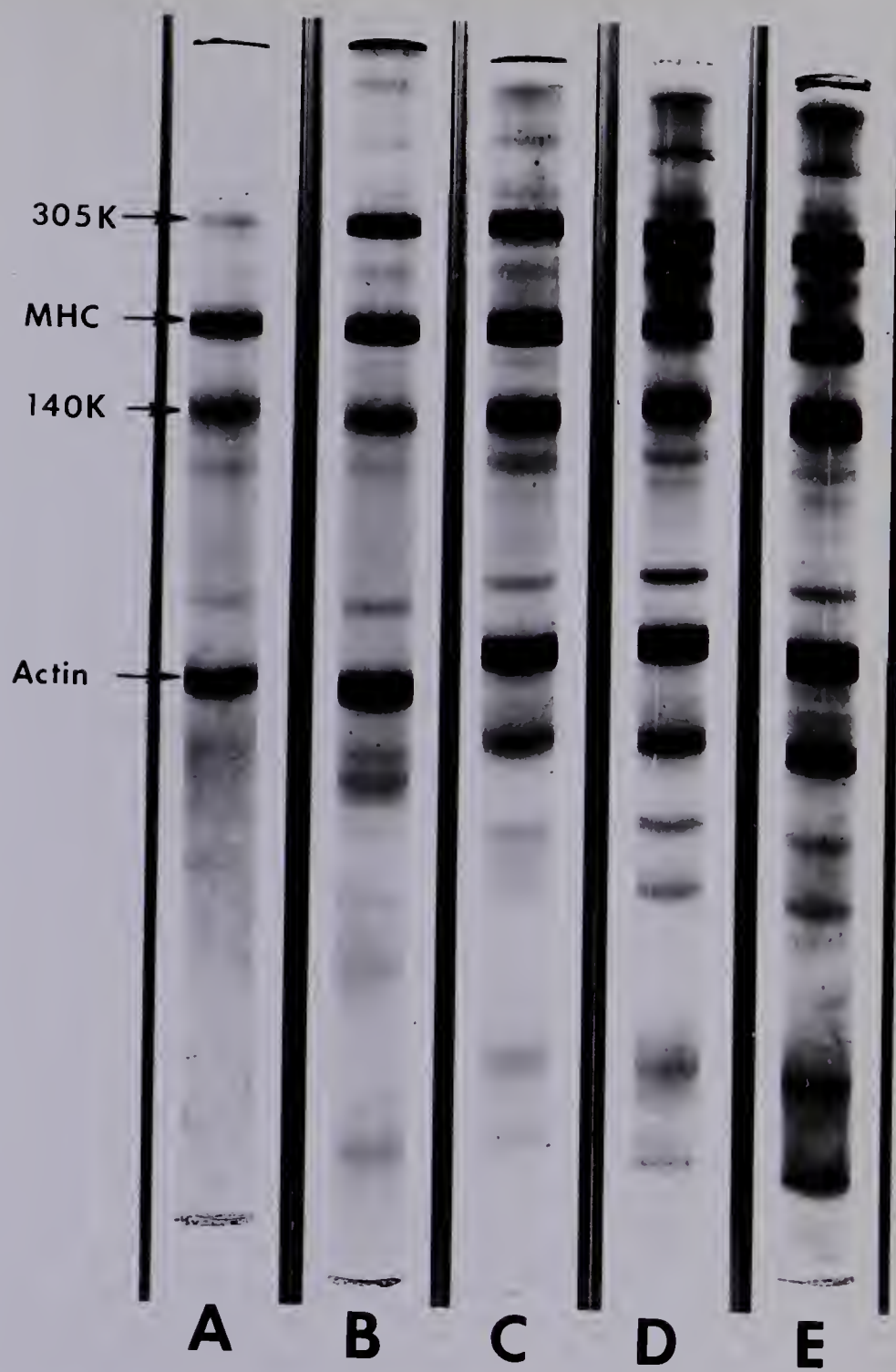
*Identification, based on molecular weights, are not conclusive.

dimer (Sender, 1971). Components (#13-16) with molecular weights in the range of 20,000 to 40,000 daltons were believed to be the regulatory proteins and myosin light chains. An unidentified protein at 12,500 daltons was consistently observed on these gels. No evidence was obtained for the presence of light chain 3 ($\sim 16,000$ daltons) or troponin C ($\sim 21,000$ daltons). It was occasionally observed that band 15, identified as light chain 1 (LC_1) migrated as a partially resolved doublet suggesting the possibility of troponin-I (24,000 daltons) in the preparation.

During the period following cell fusion in the cultures, there is an accompanying specific activation of contractile protein synthesis (Devlin and Emmerson, 1978) and assembly of the myofibril (Fischman, 1970). Consequently it was of interest to determine whether there were changes in the composition of the myofibrillar preparation, as judged by electrophoresis, during the post-fusion period. Myofibrils were therefore isolated by the method described, at various stages after fusion, and analyzed by SDS-PAGE (Figure 20).

At 84 hours post-plating the amount of material on the gels was low compared to a standard (8 day culture) preparation, with myosin heavy chain and actin being most prominent. Several other bands between myosin heavy chains and actin were also observed, but again at considerably reduced levels compared to standard preparations. Staining of the gel in the region of the regulatory proteins and light chains was faint and diffuse, and no clearly resolved bands could be discerned. The most striking feature of these gels however, was the almost complete absence of very high molecular weight

Figure 20. SDS-PAGE analysis of myofibrillar pellets obtained from cultures of normal chick embryo leg muscle at various stages of development. Cultures (3×10^6 cells/90 mm plate) were established and grown as described in Materials and Methods for 10 days with media changes on days 4 and 6. Myofibrils were isolated by the standard procedure described in Materials and Methods at a) 84 hours, b) 96 hours, c) 144 hours, d) 192 hours and e) 240 hours post plating.



proteins of more than 200 K daltons. In particular, the 305 K component, although present was greatly reduced in terms of its relative color density compared with myofibrils from later stages of development. The composition of the myofibril preparation changed during the next 12 hours, to assume a banding pattern closely resembling that of the standard preparations. Notably, the relative proportions of the 305 K component and the other very high molecular weight material were increased to levels more typical of myofibril preparations from older cultures. In addition the regulatory proteins and light chains were more clearly resolved, and there was an observable increase in the total amount of stained material on the gels. Over the next 2 days there was a further increase in the total amount of stained material, but no significant changes in the banding pattern were observed. After this stage only very slight increases in the amounts of material on the gels were noted and the banding pattern had clearly stabilized suggesting a steady state situation had been achieved.

Myofibrils prepared by the standardized method from dystrophic cultures were found to be similar in appearance to those from normal cultures, when examined by phase microscopy (Figure 21). The results of analysis of dystrophic preparations by SDS-PAGE are shown in Figure 22, and Table 11. The general features of gels of dystrophic myofibrils are similar to those from normal myofibrils in terms of the number and relative distribution of major and minor bands (see Figure 23 for a comparison). However a number of

Figure 21. Myofibrillar pellet obtained from culture of dystrophic chick embryo leg muscle using standard isolation procedures. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 8 days with media changes on days 4 and 6. Myofibrils were then isolated by the standard procedure described in Materials and Methods.

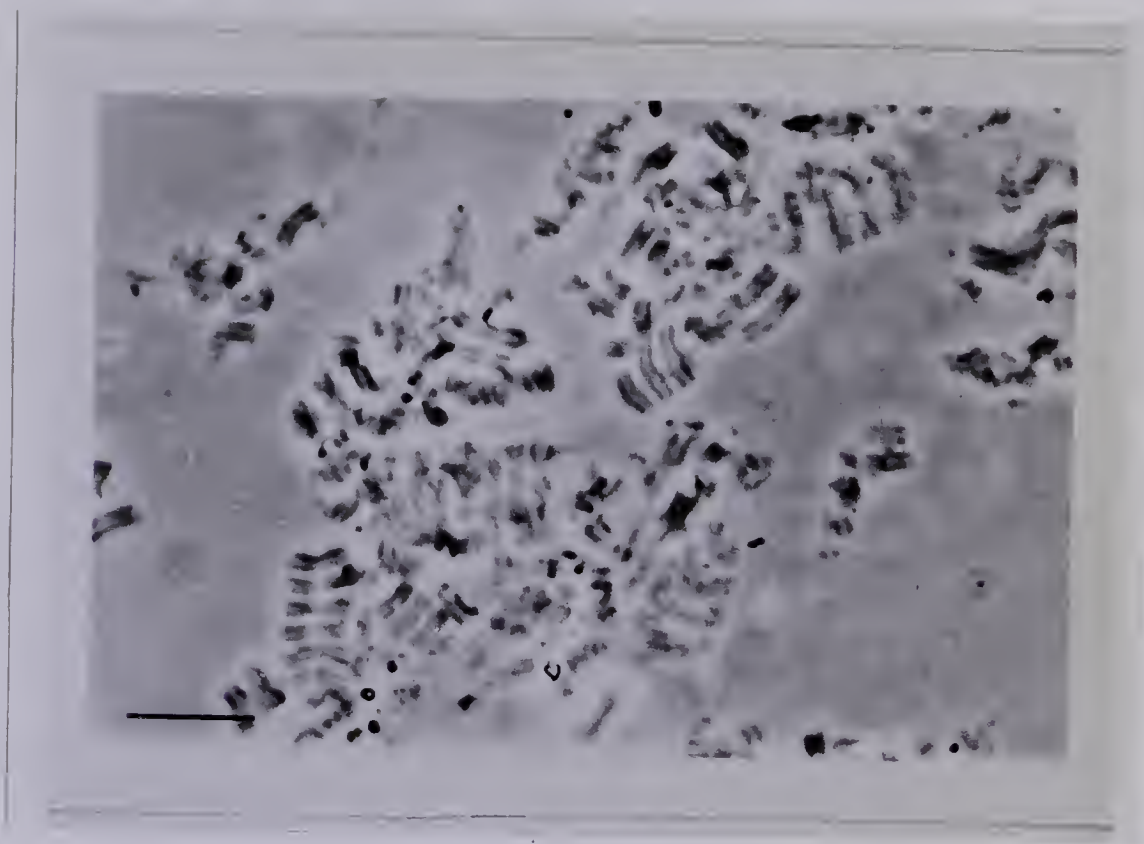


Figure 22. SDS-PAGE analysis of myofibrillar pellet obtained from cultures of dystrophic chick embryo leg muscle. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 8 days with media changes on days 4 and 6. Myofibrils were then isolated by the standard procedure and analyzed by SDS-PAGE as described in Materials and Methods.

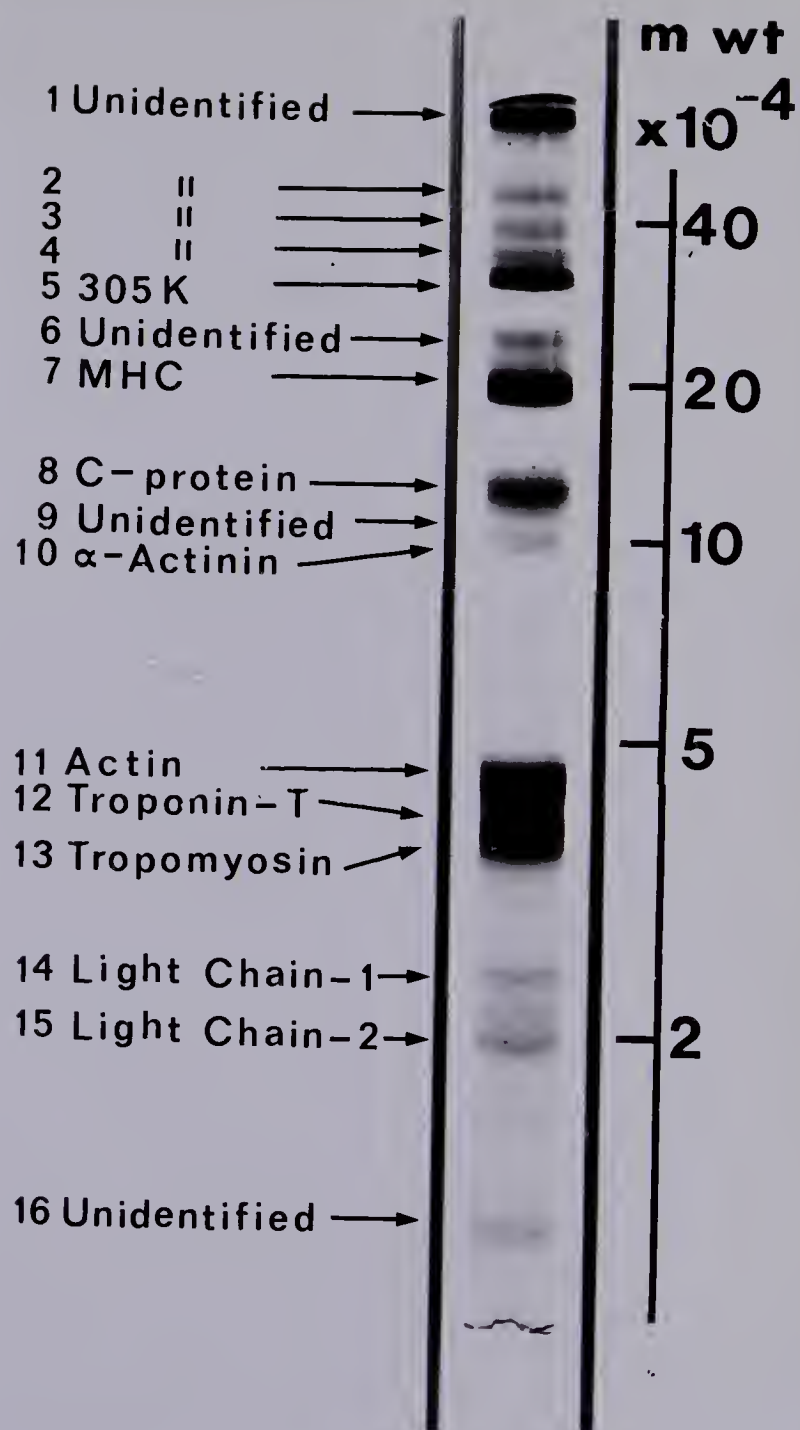


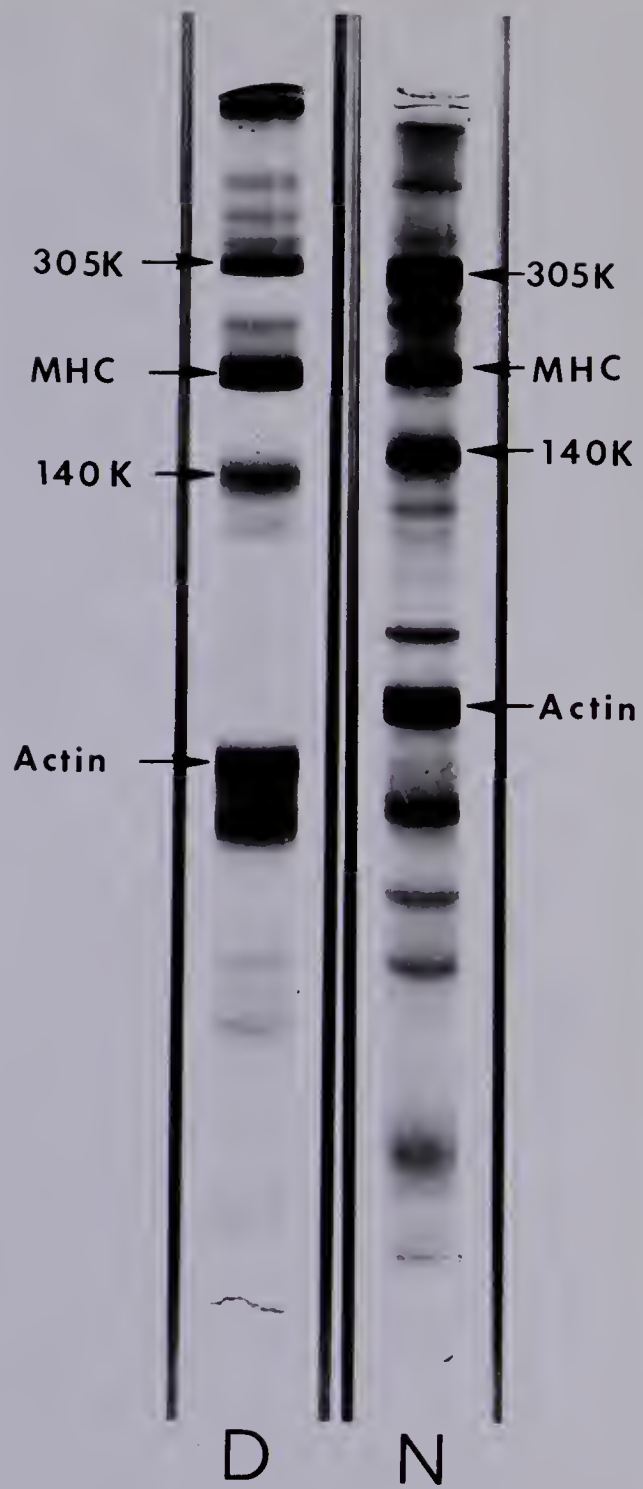
TABLE 11

COMPOSITION OF MYOFIBRILS DERIVED FROM CULTURES OF DYSTROPHIC
CHICK EMBRYO SKELETAL MUSCLE

BAND	PROTEIN*	MOLECULAR WT. (Dalton)
1	Unidentified	<500,000
2	"	<500,000
3	"	400,000
4	"	355,000
5	"	315,000
6	"	255,000
7	Myosin Heavy Chain (MHC)	200,000
8	C-Protein	140,000
9	Unidentified	110,000
10	α -Actinin	100,000
11	Actin	45,000
12	Troponin-T	38,000
13	Tropomyosin	36,000
14	Light Chain 1 (LC ₁)	26,000
15	Light Chain 2 (LC ₂)	20,000
16	Unidentified	12,500

*Identifications based on molecular weights are not conclusive.

Figure 23. Comparison of standard myofibrillar pellets obtained from cultures of normal (N) and dystrophic (D) chick embryo leg muscle. Cultures (3×10^6 cells/90 mm plate) from normal and dystrophic chick muscle were established as described in Materials and Methods and grown for 8 days with media changes on days 4 and 6. Myofibrils were then isolated and analyzed by SDS-PAGE as described in Materials and Methods.



differences were noted. First of all total amounts of protein on gels of dystrophic myofibrils were lower than in normal (Table 12 and 13). In addition the distribution and molecular weights of minor bands in the very high molecular weight region appeared to be different and a band, which probably corresponded to the 305 K component of normal myofibrils migrated with a slightly higher molecular weight (315 K). This band appeared to account for a relatively smaller proportion of the material on the gel. The doublet (110 K & 110 K) migrating below the 140 K component on dystrophic gels was not identical to that found in normal myofibrils where molecular weights of 105 K and 90 K were noted. The most obvious difference between the two types of myofibrils was the degree of resolution obtained for actin and the two regulatory proteins, troponin-T and tropomyosin. In the case of normal myofibrils actin was clearly resolved from the regulatory proteins. By contrast the resolution obtained in dystrophic preparations was much poorer and it was found that the molecular weights of these proteins were slightly different (Table 11). These results suggested a possible difference in these components between normal and dystrophic cultures. However, on co-electrophoresis of normal and dystrophic samples actin migrated as a single band.

D. Analysis of Myofibrillar Proteins

The influence of the inhibitors on the composition of the myofibrils, as judged by SDS-PAGE was investigated. Beginning with 7 day-old cultures, the inhibitors, either singly or in combination

at the concentrations previously used, were added to the medium for periods of up to 72 hours. Myofibrils were isolated and analyzed as described in the previous section. As can be seen (Figure 24) none of the inhibitor treatments produced any noticeable difference in either the banding pattern or the relative distribution of components in myofibril samples from normal cultures after 24 hours of exposure to the inhibitors. However there was a significant increase in the total amount of protein on the gels of inhibitor treated samples, and in particular with respect to the four major components (Table 12). The increase was evident at 24 hours post-labelling and was maintained through 72 hours post-labelling. In addition the extent of the increase was the same for all three inhibitor treatments examined. In parallel experiments with dystrophic cultures the composition of myofibrils was unaffected by either of the inhibitors when used on their own (Figure 25) but in contrast to the situation for normal cultures no significant increase in the amount of protein on the gels was noted (Table 13). In samples from dystrophic cultures treated with both inhibitors simultaneously for 24 hours some small differences were noted in the banding pattern (but not in the levels of the four major components (Table 13). Within the very high molecular weight region i.e. > 250 daltons, a band corresponding to 465,000 daltons was lost and a new band at 500,000 daltons developed. More noticeably the doublet of 110,000 and 100,000 daltons disappeared and there was a decrease in the relative intensity of the troponin-T/tropomyosin doublet. In addition these gels stained diffusely throughout the lower

Date	Description	Amount
1890	Jan 1 Balance	100.00
1891	Feb 1 Balance	100.00
1892	Mar 1 Balance	100.00
1893	Apr 1 Balance	100.00
1894	May 1 Balance	100.00
1895	Jun 1 Balance	100.00
1896	Jul 1 Balance	100.00
1897	Aug 1 Balance	100.00
1898	Sep 1 Balance	100.00
1899	Oct 1 Balance	100.00
1900	Nov 1 Balance	100.00
1901	Dec 1 Balance	100.00
1902	Jan 1 Balance	100.00
1903	Feb 1 Balance	100.00
1904	Mar 1 Balance	100.00
1905	Apr 1 Balance	100.00
1906	May 1 Balance	100.00
1907	Jun 1 Balance	100.00
1908	Jul 1 Balance	100.00
1909	Aug 1 Balance	100.00
1910	Sep 1 Balance	100.00
1911	Oct 1 Balance	100.00
1912	Nov 1 Balance	100.00

Figure 24. Effect of protease inhibitor treatment on composition of myofibrils from cultures of normal chick embryo leg muscle. Cultures (3×10^6 cells/90 mm plates) were established as described in Materials and Methods and grown for 7 days with media changes on days 4 and 6. Addition of inhibitors was initiated on day 7 and maintained over the following 24 hours. Myofibrils were isolated by the standard procedure described in Materials and Methods and analyzed by SDS-PAGE, a) control b) leupeptin (50 ug/ml) c) pepstatin (50 ug/ml) d) leupeptin = pepstatin (50 ug/ml).

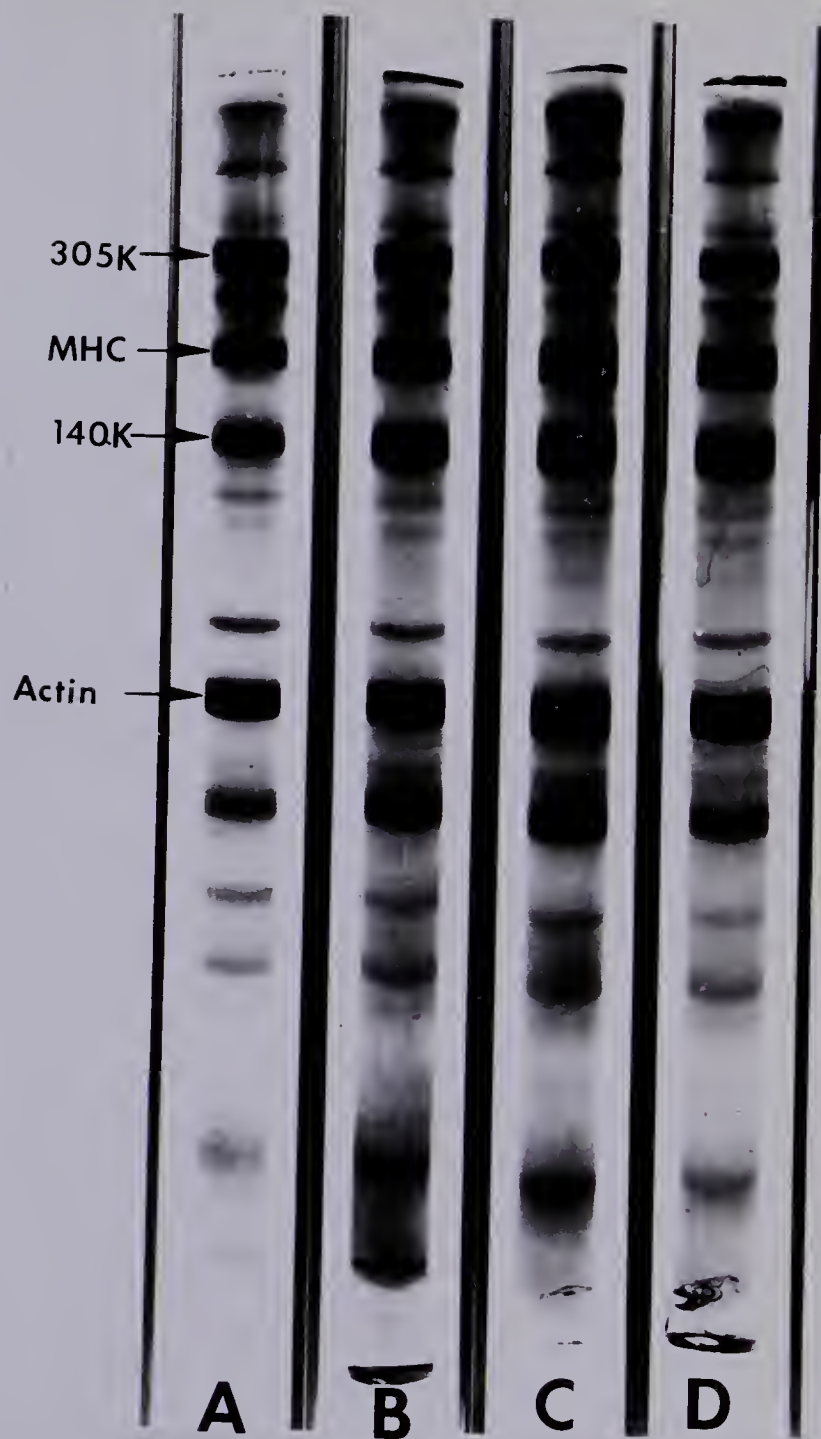


TABLE 12

Influences of protease inhibitors on levels of myofibrillar components from normal chick embryo cultures as determined from SDS-PAGE

(arbitrary units)

COMPONENT	CONTROLS			LEUPEPTIN(L)		PEPSTATIN(P)		P & L	
	0 hrs	24 hrs	72 hrs	24 hrs	72 hrs	24 hrs	72 hrs	24 hrs	72 hrs
305K	353+24	344+31	365+21	427+25	403+17	421+36	411+18	426+21	419+14
MHC	329+27	341+18	337+32	414+34	408+29	417+28	433+30	407+13	425+27
140K	270+18	256+23	242+27	332+26	319+22	316+31	297+24	359+36	321+26
ACTIN	396+24	417+27	409+33	544+26	518+33	493+21	513+38	520+27	489+27

Cultures (3x10⁶ cells/90 mm plate) were established as described in Materials and Methods and grown for 7 days with media changes on days 4 and 6. Addition of inhibitors was initiated on day 7 (0 hours) and maintained over the following 72 hours. Sample cultures were taken at 0, 24 and 72 hours, and myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Gels were quantified for protein by scanning densitometry. Values are mean +S.D. from 3 separate experiments. All treatment values for each component were significantly different from control values at p <0.01 or better. Control values for each component were not significantly different (p <0.05) at the three time points studied.

Name	Age	Sex	Occupation
John Smith	25	Male	Teacher
Mary Jones	30	Female	Nurse
Robert Brown	35	Male	Engineer
Elizabeth White	40	Female	Homemaker
William Black	45	Male	Farmer
Susan Green	50	Female	Retailer
David Lee	55	Male	Scientist
Jennifer Hall	60	Female	Librarian
Michael King	65	Male	Retired
Patricia Scott	70	Female	Volunteer
Christopher Adams	75	Male	Historian
Amanda Baker	80	Female	Artist
Daniel Clark	85	Male	Writer
Michelle Evans	90	Female	Gardener
Steven Foster	95	Male	Philosopher

Figure 25. Effect of protease inhibitor treatment on composition of myofibrils from cultures of dystrophic chick embryo leg muscle. Cultures (3×10^6 cells/90 mm plates) were established as described in Materials and Methods and grown for 7 days with media changes on days 4 and 6. Addition of inhibitors was initiated on day 7 and maintained over the following 24 hours. Myofibrils were isolated by the standard procedure as described in Materials and Methods and analyzed by SDS-PAGE.

a) control, b) leupeptin (50 ug/ml), c) pepstatin (50 ug/ml), d) leupeptin + pepstatin (50 ug.ml).

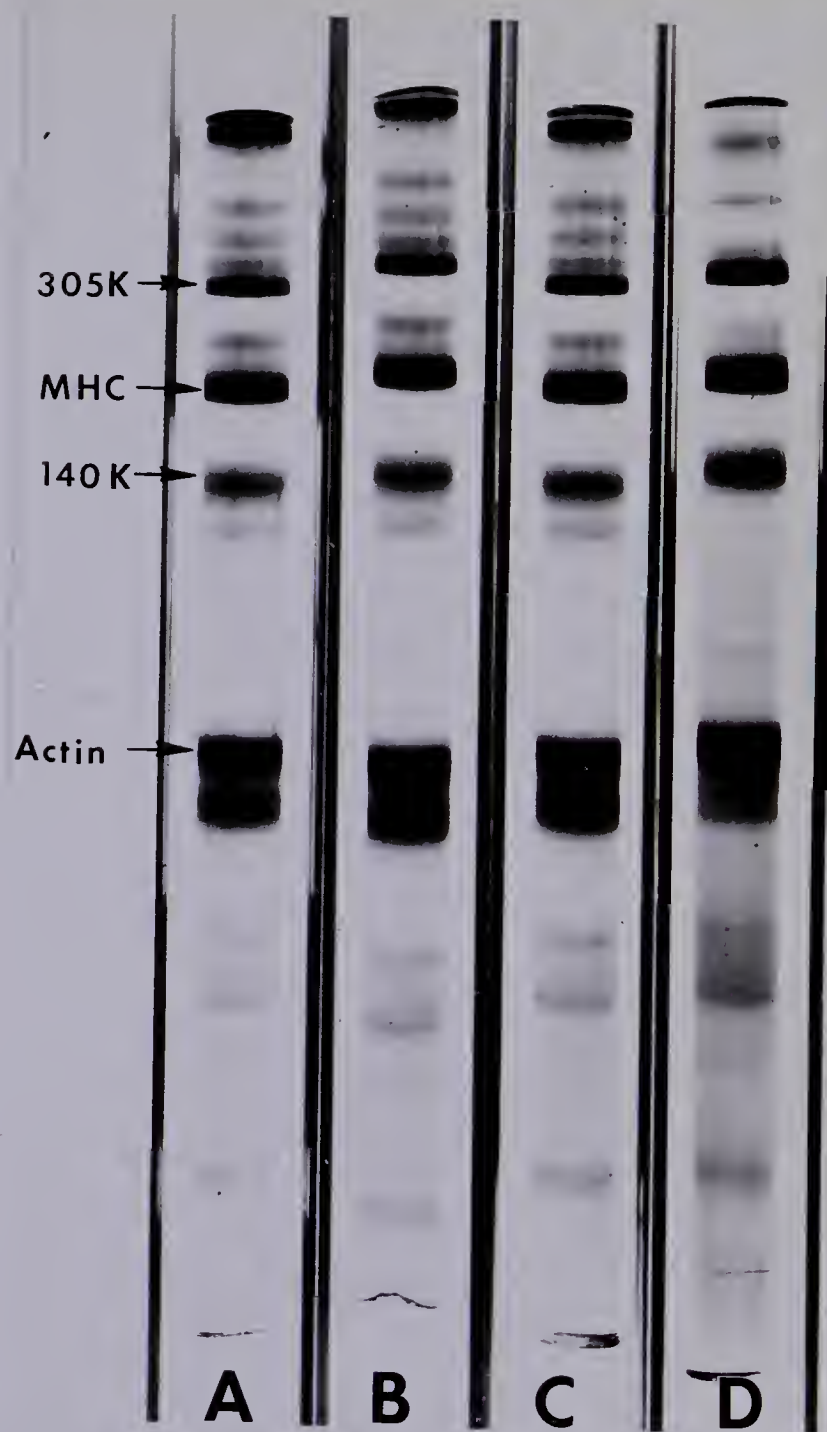


TABLE 13

Influence of protease inhibitors on levels of myofibrillar components from dystrophic chick embryo cultures as determined from SDS-PAGE.

(arbitrary units)

COMPONENT	CONTROLS		LEUPEPTIN(L)		PEPSTATIN(P)		P & L	
	0 hrs	24 hrs	72 hrs	24 hrs	72 hrs	24 hrs	24 hrs	72 hrs
305K	226+19	237+23	219+24	234+27	246+19	250+21	231+23	256+23
MHC	277+21	264+18	259+21	284+29	258+24	279+27	263+28	270+25
140K	168+14	178+18	180+11	179+16	162+16	174+19	186+18	173+15
ACTIN	347+29	356+22	340+27	352+26	346+28	358+30	367+38	360+34

Cultures (3x10⁶ cells/90 mm plate) were established as described in Materials and Methods and grown for 7 days with media changes on days 4 and 6. Addition of inhibitors was initiated on day 7 (0 hours) and maintained over the following 72 hours. Sample cultures were taken at 0, 24 and 72 hours, and myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Gels were quantified for protein by scanning densitometry. Values are mean \pm S.D. from 3 separate experiments. Treatments produced no significant differences with respect to controls at $p < 0.05$. Control values for each component were not significantly different at the three points $p < 0.05$.

molecular weight region i.e. < 50,000 daltons. Preparations of myofibrils from dystrophic cultures treated for 72 hours with both inhibitors showed a marked increase in the intensity of the diffuse staining with a concomitant decrease in the intensity of the individual bands (Figure 26).

The myofibril isolation procedure was also used to investigate the turnover of the myofibrils and the effect of the inhibitors on turnover in both normal and dystrophic cultures. Cultures were labelled between days 6 and 7 with ^{35}S -methionine and simultaneously treated with cytosine arabinoside ($5 \times 10^{-6}\text{M}$) to reduce fibroblast contamination. Myofibrils were then isolated on days 7 (0 hours), 8 (24 hours), and 10 (72 hours) and analyzed by electrophoresis. During this interval, the banding pattern and amounts of the four major components on the gels of normal controls remained constant (Table 12), suggesting that the cultures were in a steady state. Further evidence for the existence of a steady state in normal cultures during this interval was obtained from measurements of the levels of extractable protein. Thus at 0, 24, and 72 hours post-labelling, the levels of extractable protein were 11.68 ± 0.43 (0 hours), 12.19 ± 0.36 (24 hours) and 11.87 ± 0.54 (72 hours) mg. protein/90 mm. plate (the values are the mean \pm S.E. from three separate experiments). The specific activities (calculated as cpm/unit area) of the four major components on the gels were determined at the various sampling stages and decay curves were constructed from this data. Typical results obtained from normal cultures are shown in Figure 27. The initial specific activities of the four

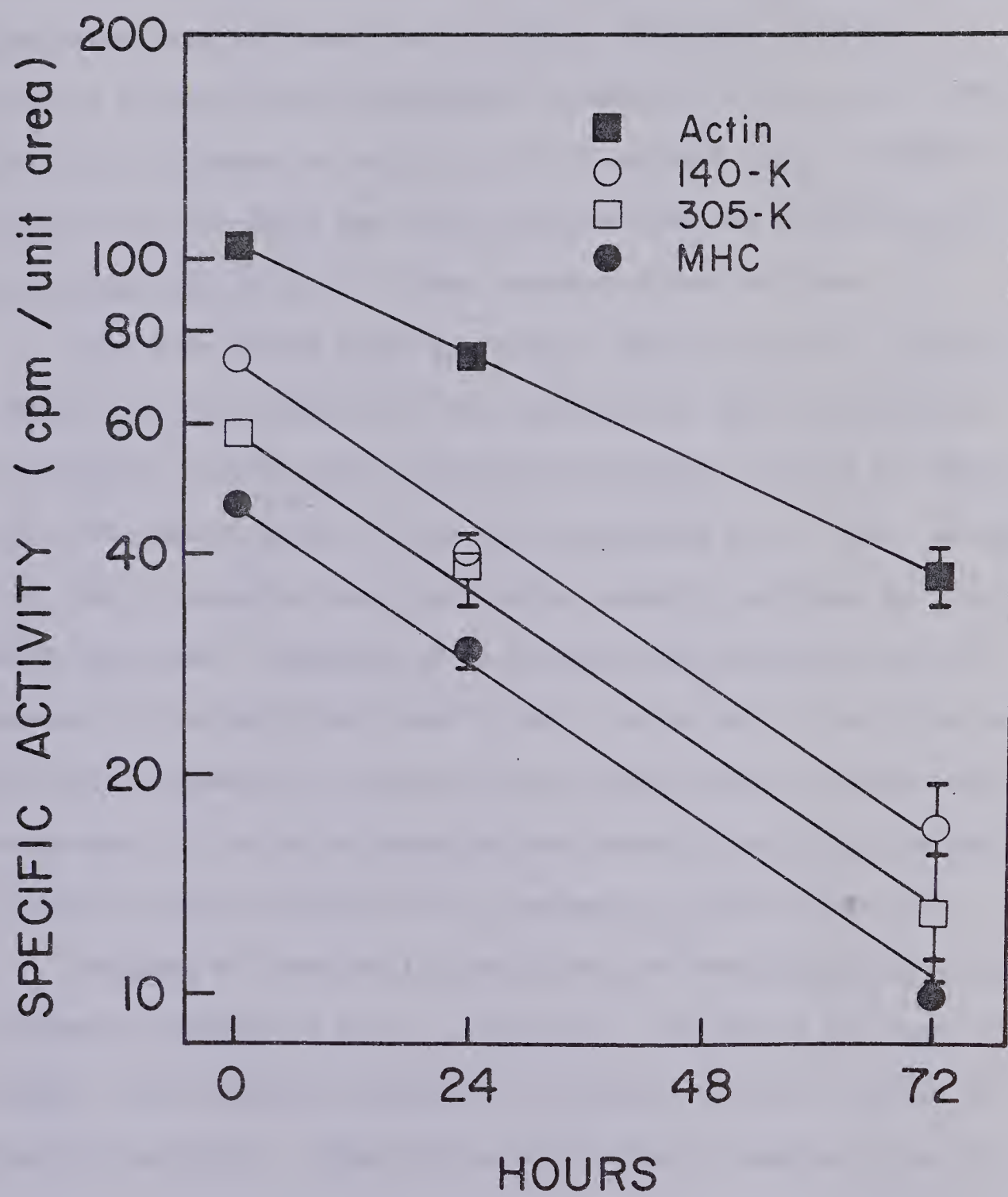
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Figure 26. SDS-PAGE analysis of myofibrillar pellet from cultures of dystrophic chick leg muscle treated for 72 hours with leupeptin plus pepstatin. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 7 days with media changes on days 4 and 6. Addition of leupeptin (50 ug/ml) plus pepstatin (50 ug/ml) was initiated on day 7 and maintained through the following 72 hours. Myofibrils were isolated by the standard procedure described in Materials and Methods and analyzed by SDS-PAGE.



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Figure 27. Specific activity decay curves of four myofibrillar proteins from cultures of normal chick embryo leg muscles. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ^{35}S -methionine (22.5 uCi/90 mm plate), between the 6th and 7th day. The end of the labelling period corresponds to 0 hours in the figure. Sample cultures were taken at 0, 24 and 72 hours post-labelling, and myofibrils were prepared and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Lines of best fit were constructed by the method of least square. Correlation co-efficients were determined as: actin (0.9993), 140K (0.99631), MHC (0.99974). Values are mean \pm S.D. (N=3 or 4).



components were found to occur in a definite and reproducible order with actin having the largest specific activity and MHC the smallest (Figure 27). Adjustment of the specific activity of actin for its methionine content relative to MHC (Devlin and Emmerson, 1978) diminished the difference to the extent that actin actually exhibited a lower rate of methionine incorporation (S.A. 35.71 cpm/unit area) compared to MHC (S.A. 47.10 cpm/unit area). Similar corrections for 305 K and 140 K proteins were not possible since the methionine content of these components was not known.

Best fits of the semi-logarithmic plots of specific activity versus time were constructed by the method of least squares and correlation co-efficients and slopes determined (Figure 27, Table 14). The decay curves for all four components were linear, indicating that turnover of these particular proteins conformed to first order kinetics. Estimates of half-lives were obtained from the slopes of the individual decay curves. Actin with a half-life of 50.3 hours appeared to turn over more slowly than the other three components all of which exhibited very similar half-lives between 35 and 39 hours throughout the experimental period (Table 14).

Patterns of turnover in the presence of the inhibitors were also determined (Figure 28 a, b, c; Table 12). In all of the cases examined, the inhibitors considerably reduced the rate of decay of specific activity. These effects were clearly apparent after 24 hours of treatment, producing inhibition in the range 45%-70%. The degree of inhibition found at 72 hours was in general lower being of the order of 20%-35%. In some cases e.g. effect of leupeptin on

TABLE 14

HALF-LIVES OF MYOFIBRILLAR PROTEINS AND THE EFFECTS OF LEUPEPTIN AND PEPSTATIN ON MYOFIBRIL
 TURNOVER IN NORMAL CHICK EMBRYO CULTURES

COMPONENT	$t_{1/2}(\text{hrs})$	$k_d \times 10^{-2}(\text{hrs}^{-1})$	% INHIBITION					
			Leupeptin		Pepstatin		Leu & Pep	
			24 hrs	72 hrs	24 hrs	72 hrs	24 hrs	72 hrs
305K	35.1+2.0	1.946	53+4.6	29+3.1	58+4.5	27+2.5	56+4.9	19+3.3
MHC	36.8+1.7	1.887	59+4.2	26+2.8 ^b	64+4.4	24+2.3 ^b	51+4.0	24+2.6 ^b
140K	38.1+1.8	1.821	62+4.8	25+3.6 ^b	48+3.8	22+2.2 ^b	63+4.7	26+2.8 ^b
ACTIN	50.3+2.2 ^a	1.384	49+4.1	31+2.2 ^b	57+4.9	28+2.3 ^b	60+3.8	32+2.5 ^b

Cultures (3x10⁶ cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ³⁵S-methionine (22.5 uCi/90 mm plate) between the 6th and 7th day. Immediately after labelling (0 hours) addition of inhibitors (50 ug/ml) was initiated and maintained over the following 72 hours. Samples were taken at 0, 24 and 72 hours post-labelling, and myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Half-lives, k_d values and % inhibition were estimated as described in Materials and Methods. Values are mean +S.E. of 3 separate experiments. a) significantly different from other 3 components p <0.01. b) significantly lower than corresponding 24 hr value at p <0.01 or better.

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Figure 28. a) Effect of leupeptin and pepstatin on specific activity curves of MHC and actin from cultures of normal chick embryos. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ^{35}S -methionine (22.5 uCi/90 mm plate) between the 6th and 7th day. The end of the labelling period corresponds to 0 hours in the figure. Addition of leupeptin (50 ug/ml) and pepstatin (50 ug/ml) was initiated on day 7 and maintained over the following 72 hours. Sample cultures were taken at 0, 24 and 72 hours post-labelling, myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Values are mean \pm S.D. (n=3 or 4). All treatments were significantly different from the controls at either 24 or 72 hours ($p < 0.001$ Students-t-test).

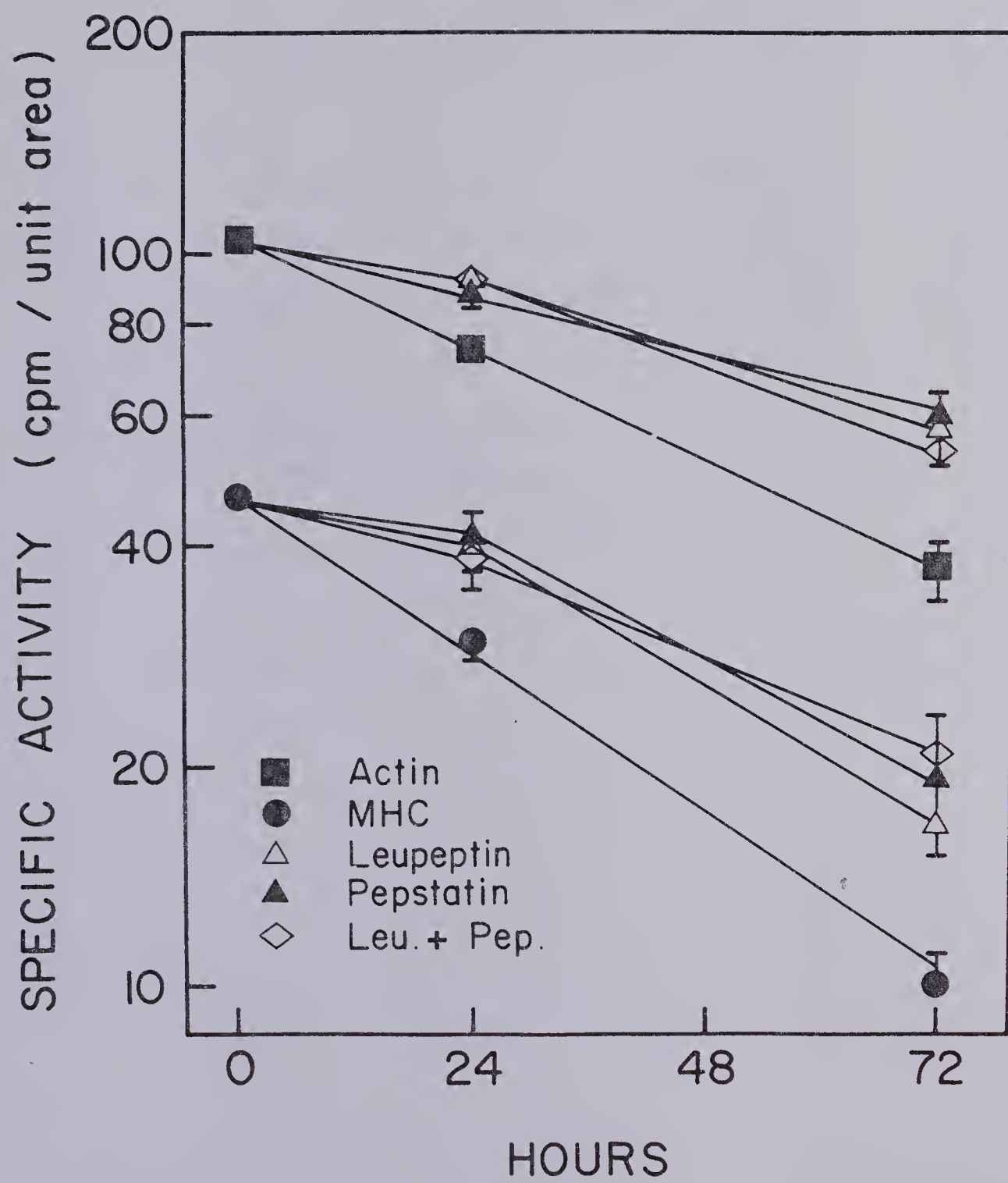


Figure 28. b) Effect of leupeptin and pepstatin on specific activity decay curves of 305K component from cultures of normal chick embryos. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ^{35}S -methionine (22.5 μCi /90 mm plate) between the 6th and 7th day. The end of the labelling period corresponds to 0 hours in the figure. Addition of leupeptin (50 $\mu\text{g/ml}$) and pepstatin (50 $\mu\text{g/ml}$) was initiated on day 7 and maintained over the following 72 hours. Sample cultures were taken at 0, 24 and 72 hours post-labelling, and myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Values are mean \pm S.D. (n=3 or 4). All treatments were significantly different from the controls at either 24 or 72 hours. ($p < 0.001$ Students-t-test).

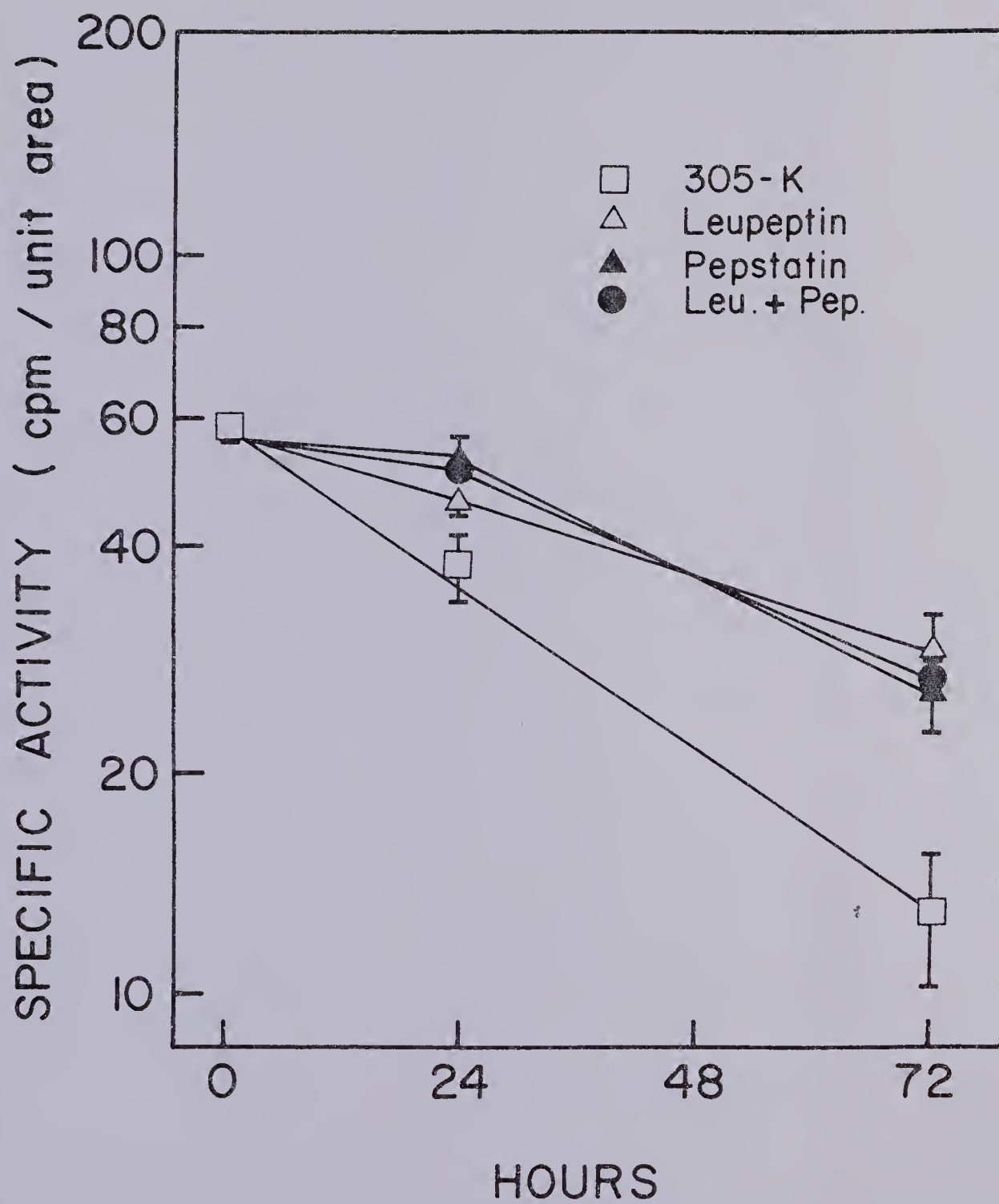
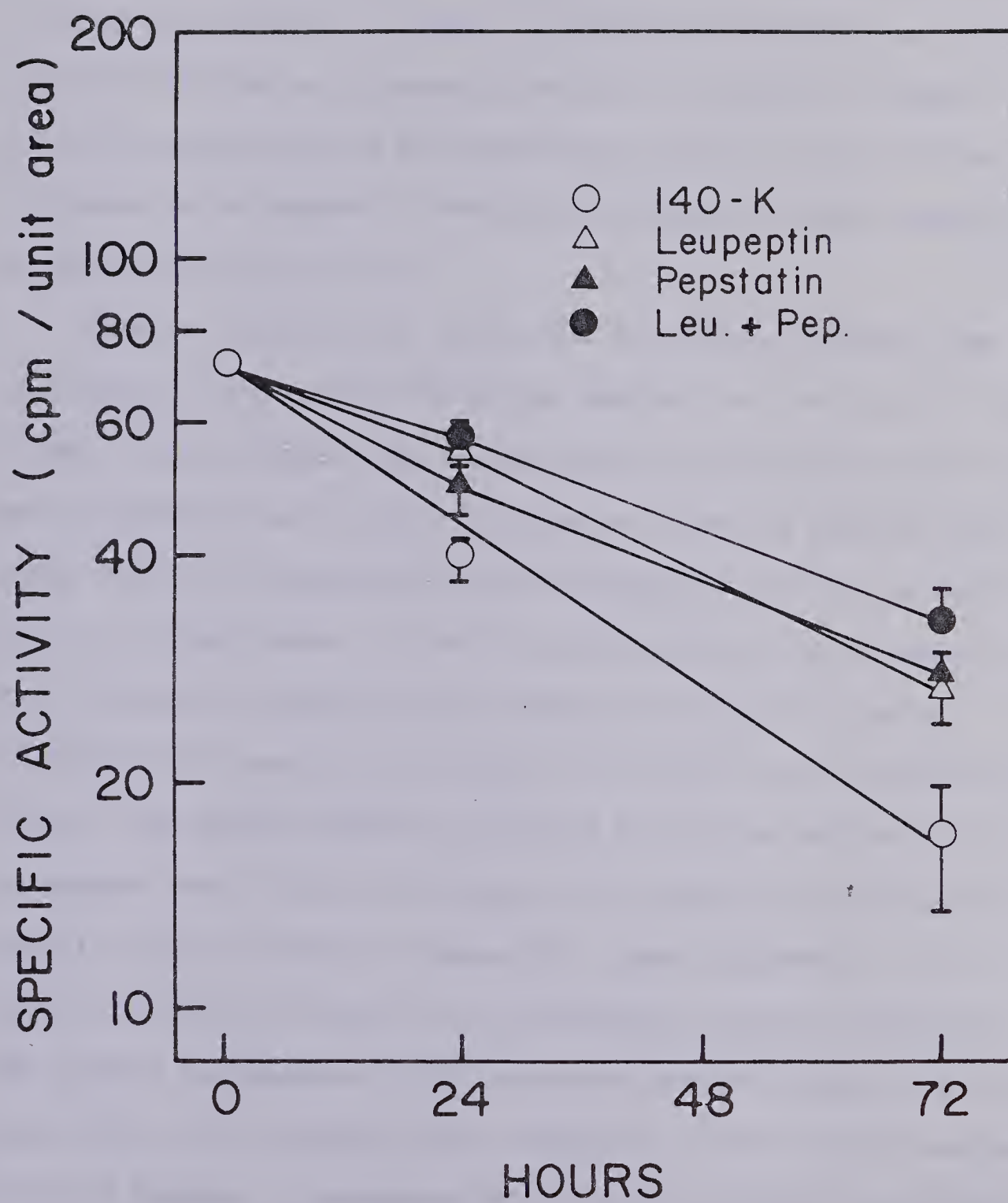




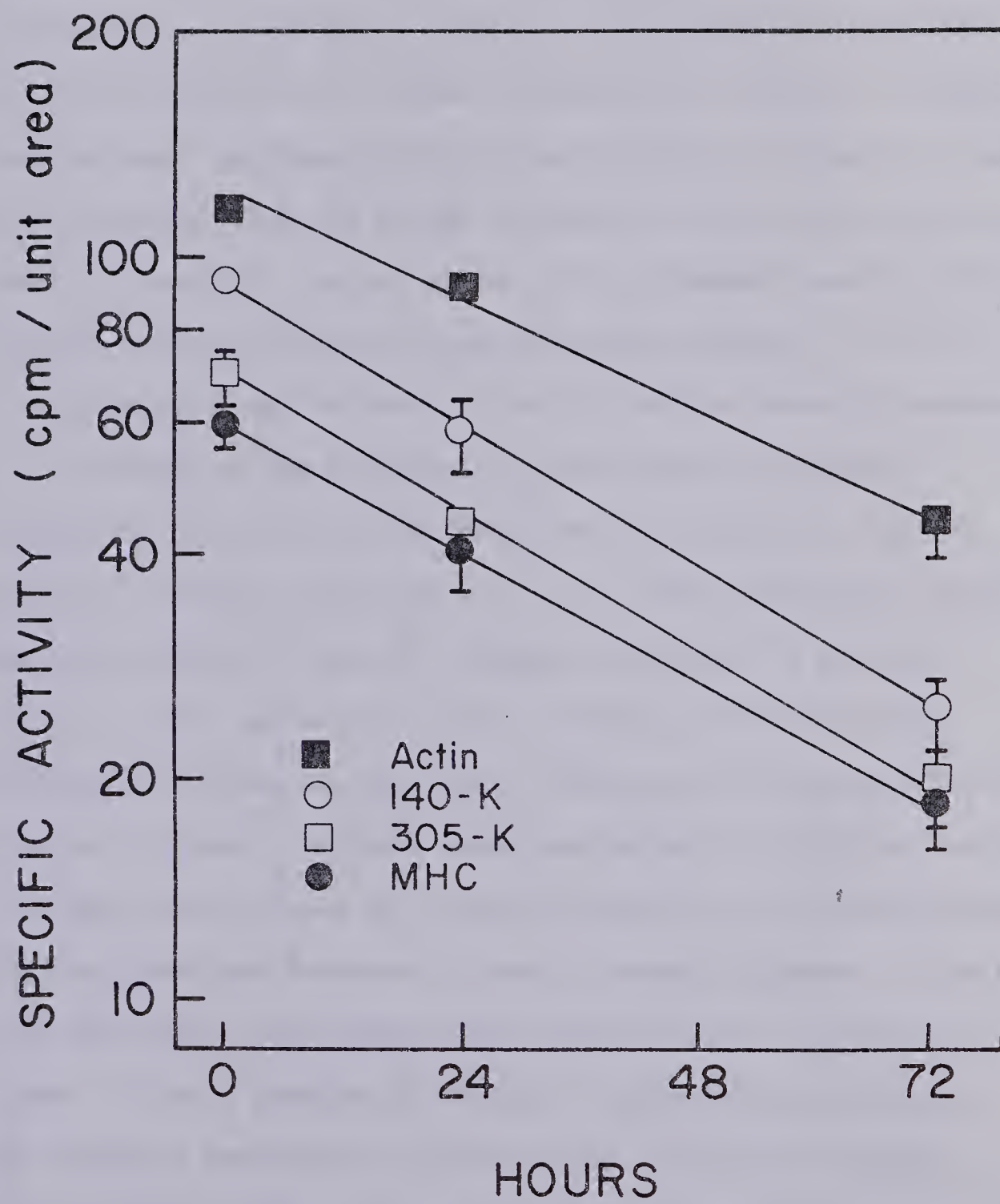
Figure 28. c) Effect of leupeptin and pepstatin on specific activity decay curves of 140K component from cultures of normal chick embryos. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ^{35}S -methionine (22.5 μCi /90 mm plate) between the 6th and 7th days. The end of the labelling period corresponds to 0 hours in the figure. Addition of leupeptin (50 $\mu\text{g/ml}$) and pepstatin (50 $\mu\text{g/ml}$) was initiated on day 7 and maintained over the following 72 hours. Sample cultures were taken at 0, 24 and 72 hours post-labelling, myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Values are mean \pm S.D. (n=3 or 4). All treatments were significantly different from the controls at either 24 or 72 hours ($p < 0.001$ Students-t-test).



MHC, the decay curves during the 24 to 72 hour interval appeared to be parallel to the controls suggesting that turnover had been restored to control levels during this period. The results also indicated that leupeptin and pepstatin, at the concentrations used, were equally effective. However, unlike the situation found in analysis of total cell protein there was no evidence to suggest that the combined use of the inhibitors resulted in any further increases in the degree of inhibition produced by either leupeptin or pepstatin on their own.

Parallel studies were carried out on cultures prepared from the muscle of genetically dystrophic embryos. As was found for the normal control cultures the banding pattern and amounts of the four major components on the gels of dystrophic controls remained constant during the experimental period suggesting that the cultures were in a steady state. In addition the levels of extractable protein during this interval were constant (11.71 ± 0.41 (0 hours), 12.46 ± 0.56 (24 hours), and 12.23 ± 0.49 (72 hours) mg. protein/90 mm plate. The initial specific activities of the four myofibrillar components were found to be ranked in the same order as that for normal cultures (Table 13, Figure 29). Again adjustment of the specific activity of actin for its methionine content relative to MHC (Devlin and Emmerson, 1978) diminished the difference such that actin (S.A. 38.90 cpm/unit area) exhibited a lower rate of incorporation of isotope. A comparison of the initial specific activities of the four components shown in Figure 27 and 29 indicates that

Figure 29. Specific activity decay curves of four myofibrillar proteins from cultures of dystrophic chick embryo leg muscles. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ^{35}S -methionine (22.5 μCi /90 mm plate) between the 6th and 7th day. The end of the labelling period corresponds to 0 hours in the figure. Sample cultures were taken at 0, 24 and 72 hours post-labelling, and myofibrils were prepared and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Lines of best fit were constructed by the method of least square. Correlation co-efficients were determined as: actin (0.99427), 140K (0.99952), 305K (0.99976), MHC (0.99980). Values are mean \pm S.D. (n=3 or 4).



dystrophic myofibrillar proteins incorporated more isotope during the labelling period. However these differences were within experimental variation and were not significantly different suggesting that the specific rates of synthesis were in fact similar in both normal and dystrophic cultures. Semi-logarithmic plots for the four components were linear indicating that turnover of these proteins also conformed to first order kinetics. Estimates of half-lives obtained from the slopes (cpm/hour) of the decay curves are shown in Table 15. The half-lives of the dystrophic myofibrillar proteins were not found to be significantly different ($p < 0.05$) from those of normal cultures indicating similar rates of turnover.

Treatment of the dystrophic cultures with the protease inhibitors resulted in an inhibition of the turnover of the myofibrillar proteins (Figure 30 a, b, c). These effects were readily detectable after 24 hours of treatment and were of a magnitude similar to that produced in normal cultures at the same stage. Differences between the dystrophic controls and treatments were also noted at 72 hours. In these cases the degree of inhibition was both less than that produced at 24 hours (similar to the normal cultures) and less than that found at 72 hours in normal cultures. In addition the slope of the decay curves between 24 and 72 hours were in almost all cases parallel to controls, suggesting that turnover may have been restored to control levels during this interval. Similar observations were made for normal cultures but as already mentioned it appeared to be a less frequent occurrence in these

TABLE 15
HALF-LIVES OF MYOFIBRILLAR PROTEINS AND THE EFFECTS OF LEUPEPTIN AND PEPSTATIN ON MYOFIBRIL
TURNOVER IN DYSTROPHIC CHICK EMBRYO CULTURES

COMPONENT	$t_{1/2}$ (hrs)	$k_d \times 10^{-2} (\text{hrs}^{-1})$	% INHIBITION					
			Leupeptin		Pepstatin		Leu & Pep	
			24 hrs	72 hrs	24 hrs	72 hrs	24 hrs	72 hrs
305K	37.4+15	1.8541	57+4.3	14.6+1.3	61+3.6	17.6+1.3	48+3.6	--
MHC	39.7+1.6	1.7466	58+4.0	12.8+0.9	52+3.9	11.7+0.8	54+2.9	--
140K	40.6+1.6	1.7080	48+3.8	15.8+1.1	63+3.2	18.0+1.0	46+3.7	--
ACTIN	53.6+1.3a	1.2939	50+4.4	12.9+1.2	54+4.5	13.4+0.9	57+4.1	--

Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with 35g-methionine ($22.5 \text{ uCi}/90\text{mm plate}$) between the 6th and 7th day. Immediately after labelling addition of inhibitors (50 ug/ml) was initiated and maintained over the following 72 hours. Samples were taken at 0, 24 and 72 hours post-labelling, and myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gel were quantified for both protein and radioactivity and specific activities determined. Half-lives k_d values and % inhibition were estimated as described in Materials and Methods. Values are mean \pm S.E. of 3 separate experiments.

- a) significantly different from other 3 components $p < 0.01$.
- b) significantly lower than corresponding 24 hour value at $p < 0.01$.

Figure 30. a) Effect of leupeptin and pepstatin on specific activity decay curves of MHC and actin from cultures of dystrophic chick embryos. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ^{35}S -methionine (22.5 uCi/90 mm plate) between the 6th and 7th day. The end of the labelling period corresponds to 0 hours in the figure. Addition of leupeptin (50 ug/ml) and pepstatin (50 ug/ml) was initiated on day 7 and maintained over the following 72 hours. Sample cultures were taken at 0, 24 and 72 hours post-labelling, myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Values are mean \pm S.D. (n= 3 or 4). All treatments were significantly different from the controls at either 24 or 72 hours. (p <0.001 Students-t-test).

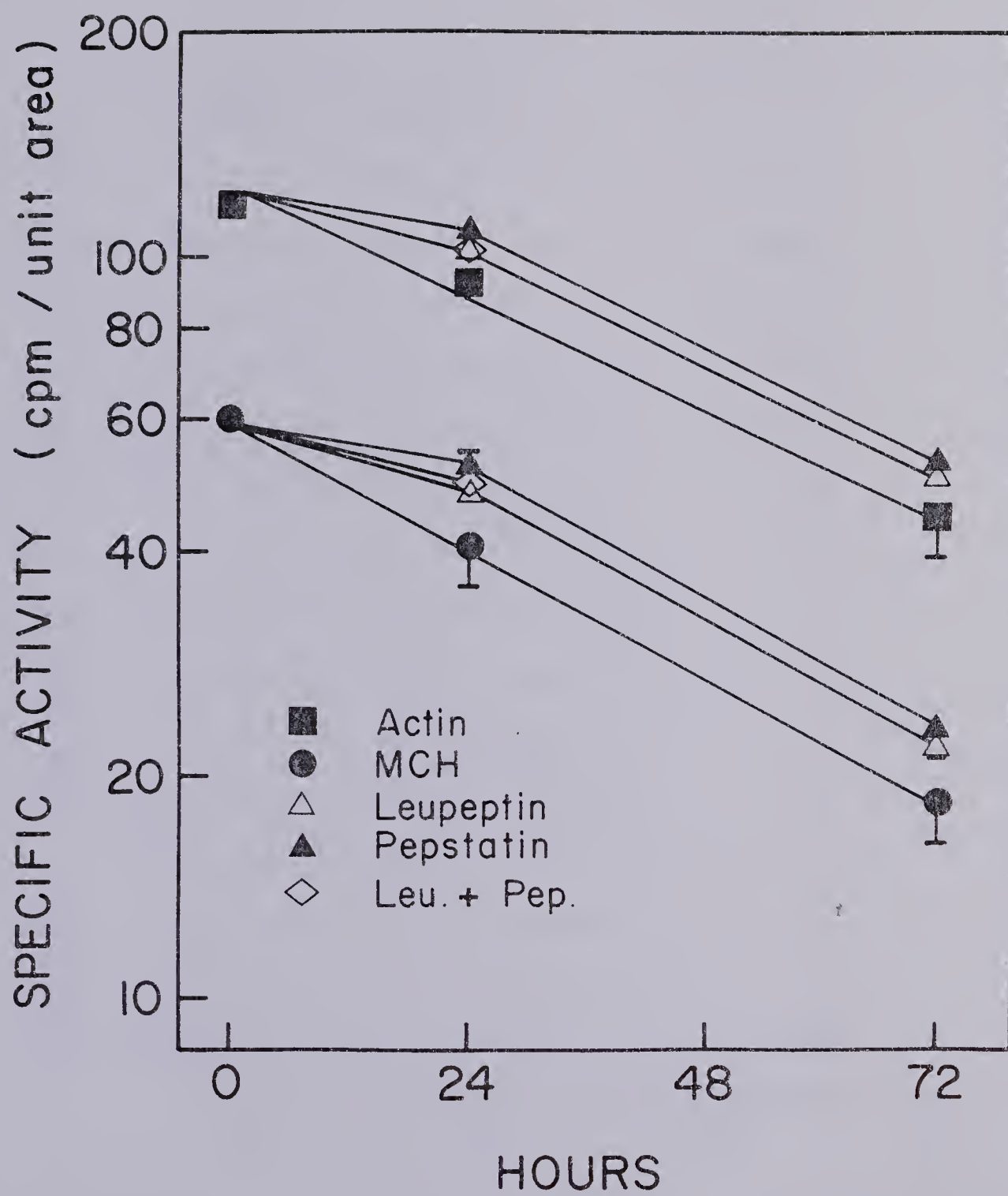




Figure 30. b) Effect of leupeptin and pepstatin on specific activity decay curves of 305K component from cultures of dystrophic chick embryos. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ^{35}S -methionine (22.5 uCi/90 mm plate) between the 6th and 7th day. The end of the labelling period corresponds to 0 hours in the figure. Addition of leupeptin (50 ug/ml) and pepstatin (50 ug/ml) was initiated on day 7 and maintained over the following 72 hours. Sample cultures were taken at 0, 24 and 72 hours post-labelling, myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Values are mean \pm S.D. (n=3 or 4). All treatments were significantly different from the controls at either 24 or 72 hours. ($p < 0.001$ Students-t-test).

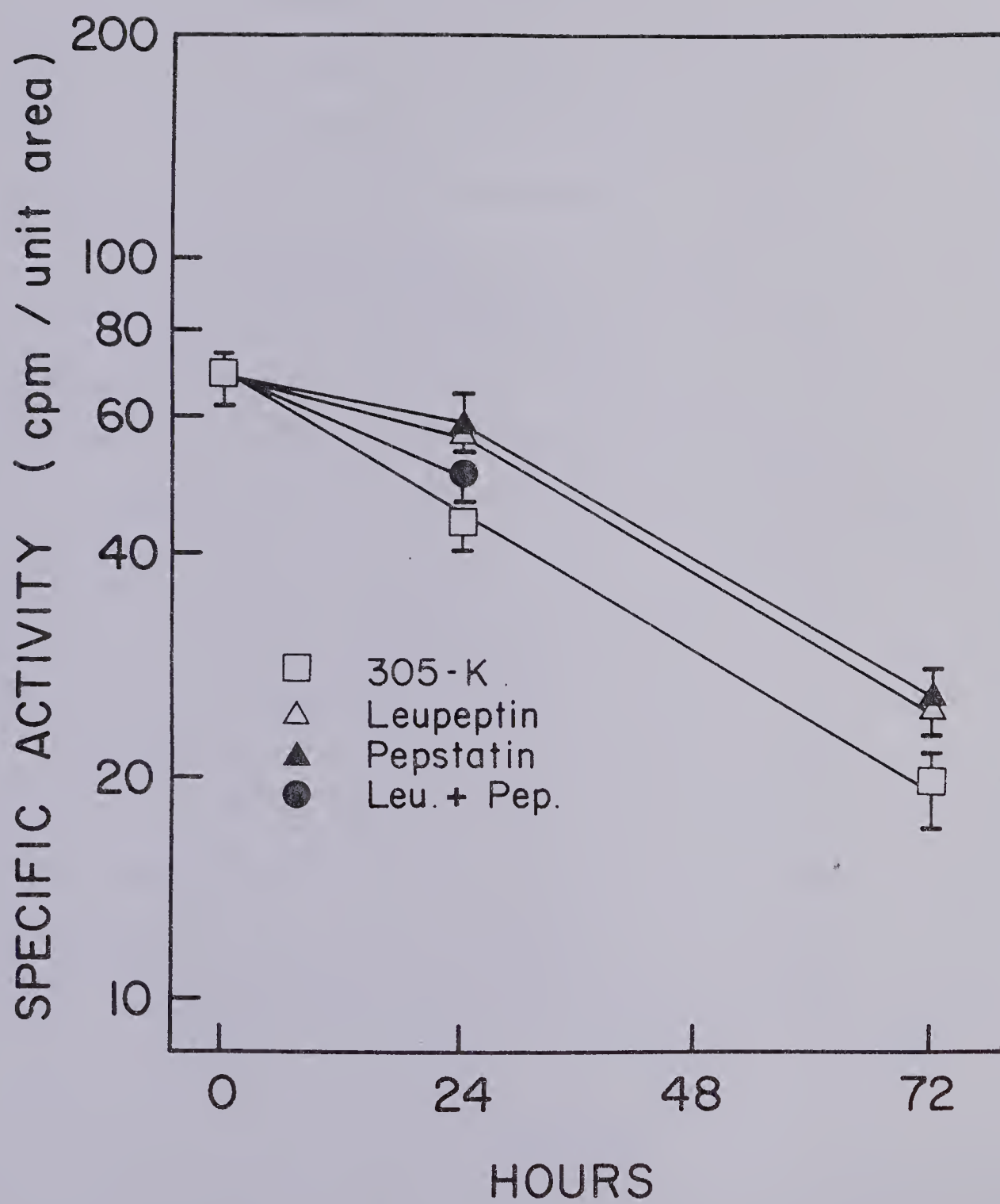
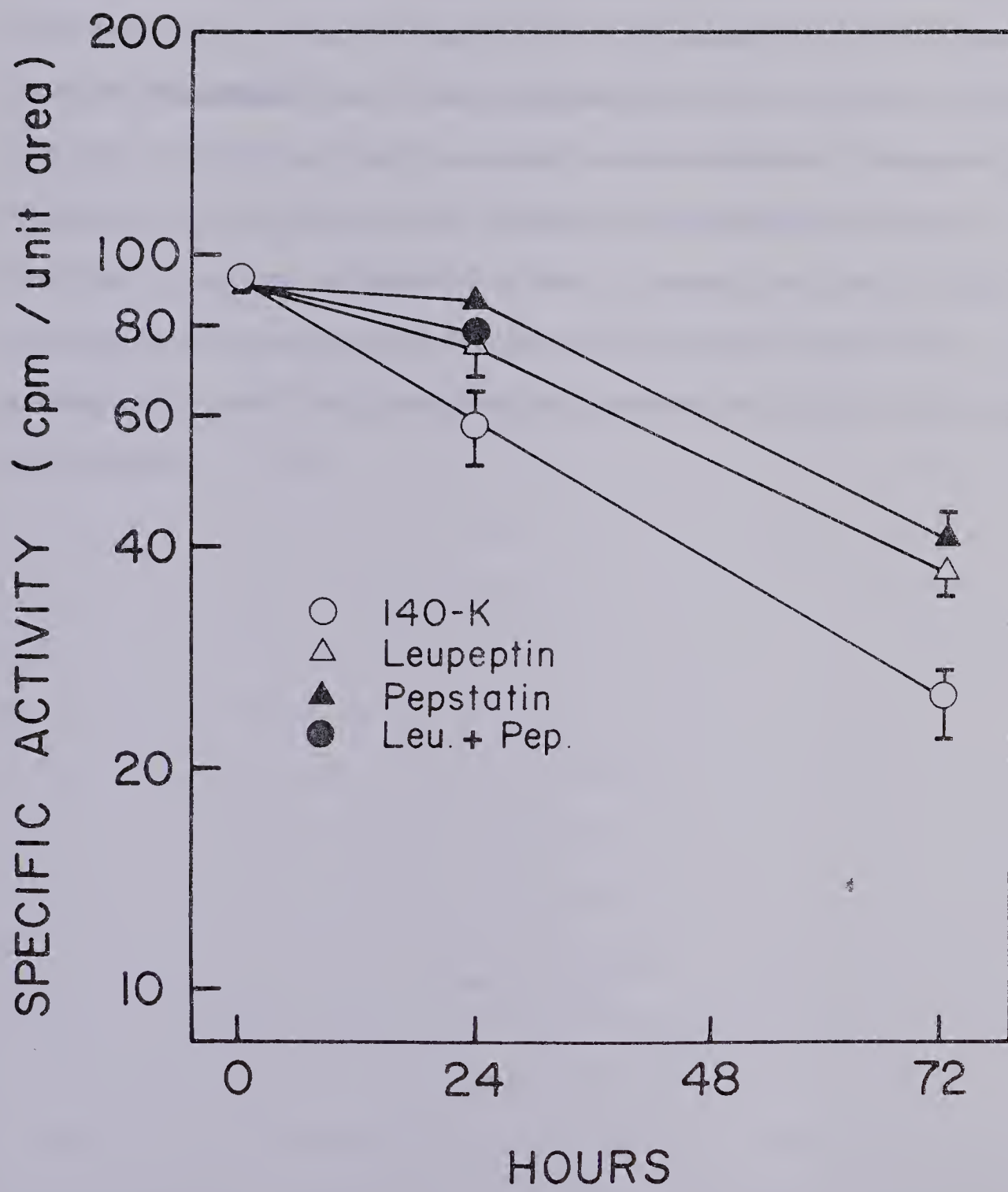


Figure 30. c) Effect of leupeptin and pepstatin on specific activity decay curves of 140K component from cultures of dystrophic chick embryos. Cultures (3×10^6 cells/90 mm plate) were established as described in Materials and Methods and grown for 6 days with a medium change on day 4. Cultures were labelled with ^{35}S -methionine (22.5 uCi/90 mm plate) between the 6th and 7th day. The end of the labelling period corresponds to 0 hours in the figure. Addition of leupeptin (50 ug/ml) and pepstatin (50 ug/ml) was initiated on day 7 and maintained over the following 72 hours. Sample cultures were taken at 0, 24 and 72 hours post-labelling, myofibrils were isolated and analyzed by SDS-PAGE as described in Materials and Methods. Individual bands on gels were quantified for both protein and radioactivity and specific activities determined. Values are mean \pm S.D. (n=3 or 4). All treatment were significantly different from the controls at either 24 or 72 hours. ($p < 0.001$ Students-t-test).



cases. No evidence was obtained to indicate that either one of the inhibitors was consistently more effective than the other, and no clear additive effect could be distinguished when the inhibitors were used in combination. Specific activity values for samples from dystrophic cultures treated with both inhibitors for 72 hours were not determined due to the degenerating state of these cultures, and the inability to obtain acceptable electrophoresis preparations. Finally, it was noted that the effect of the inhibitors on the dystrophic cultures differed from that in normal cultures, in that no significant increases were found in the levels of the four components in myofibrils obtained by standard procedures from treated cultures.

V. DISCUSSION

A. General Considerations

The primary objective of the studies presented here was to obtain information on the mechanism(s) of protein degradation in skeletal muscle, and in particular the mechanism(s) by which the myofibril is degraded. This somewhat neglected aspect of muscle biology was of interest for several reasons. In the first place the capacity to synthesize and degrade proteins continually, appears to be a general property of all cells, not just muscle (Schimke, 1975; Kay, 1978; Waterlow et al., 1978). As such, an understanding of how this fundamental process works and influences the behavior of cells in general and the particular characteristics it assumes in specific tissues such as muscle, would constitute an important contribution to the field of cell biology. Secondly, skeletal muscle, by virtue of its mass and protein content, has a position of central significance in the overall protein metabolism of animals (Young, 1970), and in this context would be of relevance to both agriculture and medical scientists. In order to define more precisely this metabolic role of skeletal muscle, a clearer picture of the underlying cellular (and subcellular) events is essential. Similarly the well recognized capacity of skeletal muscle to adapt in response to various stimuli (as for example during states of hypertrophy and atrophy), may well be governed by the activity of the proteolytic mechanisms of the tissue and again an understanding of these mechanisms would be of value in the fields of both medicine

and agriculture. Of particular interest are the muscular dystrophies which characteristically exhibit a gradual loss of protein from the muscle and occur in both humans (Monckton, 1978) and animals (Telford, 1971). At present the relationship between the primary lesion and the net catabolism of the muscle is unknown. Neither is it clear whether this net catabolism is the result of a relative increase in the normal proteolytic mechanisms of muscle or whether processes distinct to the pathological state are responsible. Information on the breakdown of the myofibril is essential since this organelle accounts for the bulk of muscle proteins. In addition an understanding of this process may be of more general significance in terms of the turnover of other complex intracellular structures such as mitochondria or membrane systems.

In deciding upon an approach to this problem, it was noted that the preliminary studies of Dean (1975b), in which he used pepstatin to inhibit proteolysis in the perfused rat liver, illustrated the potential of the microbial protease inhibitors as tools for the investigation of intracellular protein degradation. They appeared to be relatively non-toxic thus making them preferable to the synthetic protease inhibitors such as iodoacetamide or phenylmethylsulphonylfluoride. Furthermore, their high degree of specificity could conceivably permit at least tentative identification of intracellular sites of action and from this information it may in turn be possible to identify functional components of the degradative machinery. Also, successful inhibition of protein degradation

might result in the accumulation of proteolytic intermediates allowing for the elucidation of degradation pathways for individual proteins or groups of proteins such as the myofibril.

Based on these considerations, a study of the effects of leupeptin and pepstatin on protein breakdown in skeletal muscle was proposed. Although it would have been desirable to carry out these studies in vivo, the technical difficulties involved in specifically directing the inhibitors to the muscle, precluded this approach and a suitable in vitro model was therefore sought. In selecting such a model, a number of basic criteria that were considered important were defined. First it was felt that the model should contain a high proportion of well differentiated muscle fibres as free as possible from contamination by non-muscle cell types. Secondly, the inhibitors should have good access to all the cells in the preparation. This would not only facilitate their uptake but it would also ensure more uniform exposure of all cells to the inhibitors thus avoiding the possibility of untreated regions which could give rise to inconsistencies and sources of variation. In addition, the system should be capable of surviving, in a healthy state, for periods of several days and possibly weeks. This characteristic was thought important since muscle proteins are known to turnover relatively slowly (Waterlow et al., 1978). Consequently any effects of the inhibitors may require periods of several days before they become detectable. A final consideration was that the system should be amenable to the analytical techniques required to

investigate the effects of the inhibitors on protein degradation. Specifically it should be possible to carry out biosynthetic labelling and to isolate the labelled proteins for subsequent analysis by a variety of techniques.

In the experiments carried out by Libby and his colleagues (Libby and Goldberg, 1978; Libby et al., 1979), skeletal and cardiac muscle preparations derived from growing rats and fetal mice were used. Neither of these preparations were entirely suited to my needs. Both types of preparations contained a proportion of non-muscle cells, although the skeletal muscle preparations were chosen so as to be homogenous for muscle fibre type. In addition uniform uptake of the inhibitors was almost certainly dependent upon diffusion since these preparations were composed of tissue pieces several cell layers thick. This problem may have been particularly relevant to the skeletal muscle preparations, since they were only capable of being maintained in vitro for a few hours, a period which may not have been sufficient to allow for adequate penetration by the inhibitors. The fetal cultures on the other hand could be maintained for several days. However as pointed out by Libby et al., (1979) the tissue exhibited a very strong negative nitrogen balance and atrophied quite markedly throughout the experimental period. The state of health of this preparation and its value as a model for studies of normal intracellular protein degradation is therefore questionable. Another problem with these systems was that the results were subject to variations between animals, since only one or at most two (contralateral muscles) preparations could be obtained

from a single animal. Finally, proteolysis was estimated from the rate of release of tyrosine into the incubation medium, and inhibition was evaluated from significant reductions in this rate. It is however conceivable that reductions in the rate of tryosine release could have resulted from the action of the inhibitors on processes other than the proteolytic mechanisms of the cell. Even if this were not true, measurement of tryosine release could only serve as an estimate of the total protein degradation in the cell and could in no way be used to study the breakdown of individual proteins such as the myofibrillar proteins. A more direct measurement of proteolysis involving the proteins themselves would be preferable. For these reasons alternative in vitro models of skeletal muscle were sought.

From the point of view of the need for long term maintenance, the choice of systems was actually quite limited. The only systems which appeared capable of satisfying this requirement were the tissue culture systems, the most accessible of which was that derived from chick embryos (Hauschka, 1972). In recent years muscle tissue culture has been used extensively as an in vitro model of myogenesis (Hauscha, 1972; Konigsberg, 1963; Holtzer et al., 1975). Consequently most of the studies carried out on these systems concentrated on events surrounding the fusion process which occurs during the early stages of culture (between 48 and 72 hours in the system used in this study). The system has attracted considerably less interest as a model for studies of differentiated muscle. However it is

apparent from the developmental work that under appropriate conditions the embryonic cells attach and grow as a monolayer to form multinucleated, cross-striated myotubes, which are capable of survival for periods of up to several weeks (Hauschka, 1972). That these cells can remain healthy for most of this period is suggested not only by their morphological appearance but also by their ability to incorporate labelled precursors into the cellular proteins indicating an active synthetic machinery (Devlin and Emmerson, 1978).

There were however a number of disadvantages to the culture system, some of which were overcome during the course of this study. First of all in very general terms, it is important to bear in mind that cultured muscle exists in an environment which is radically different from its natural state, and that extrapolation of results from this system to muscle in vivo can only be made with extreme caution, if at all. Among the many differences which exist, the absence of an anatomically distinct innervation and a normal blood supply, may be fundamental in producing important differences between muscle in vivo and that maintained in vitro. More specifically the presence of contaminating non-myogenic tissue, is a chronic problem which becomes increasingly important with the age of the culture. This difficulty can be overcome to some extent by the use of preplating techniques and drugs such as cytosine arabinoside which specifically eliminates actively dividing cells (Fischbach, 1972; Dryden, Erulkar and de la Haba, 1974). It should however be

stressed that in evaluating data from these cultures the influences of possible contributions from this source should be taken into consideration. From a practical point of view, it was anticipated that the very small amounts of cellular material present in a single culture, could give rise to problems of obtaining sufficient quantities of material for analysis. In particular the prospects of obtaining a reasonably pure preparation of myofibrils were not altogether certain. Although this difficulty was eventually resolved in a fairly straightforward manner it proved to be one of the most difficult problems encountered during the course of the study.

In view of the ultimate objectives of the study ie. to investigate myofibrillar degradation, initial experiments were carried out in order to optimize culture conditions for the development and long term maintenance of well differentiated, cross-striated, contracting myotubes. The use of initial plating densities as high as 12×10^6 cells/90 mm plate resulted in cultures which underwent rapid differentiation, but exhibited poor long term survival, and in general tended to become over-grown by fibroblasts after a few days. It was not therefore possible to increase the amount of muscle in the cultures, by increasing the size of the initial inoculum. Optimal plating densities, in terms of development, survival and lack of contamination were found to be about $3-6 \times 10^6$ cells/90 mm plate. Cultures prepared in this way were essentially identical, therefore a plating density 3×10^6 cells/90 mm plate was used throughout to minimize the amount of starting

material necessary. Several variations of the media composition were also tested. The most important factor found in this respect was the batch of horse serum used. Considerable variation in the ability of different batches to support myogenesis and maintain differentiated muscle was noted (Hauschka, 1972). Consequently routine screening of several batches of serum was carried out before selecting the best for use in experiments. As far as it was possible the same batch of serum was used throughout any given experiment to minimize this as a source of variation. With regard to the other components of the medium, rooster serum was used because of its reported value as a source of "muscle trophic factor," shown by Ozawa (1977, 1978a, 1978b), to be effective in the maintenance of differentiated chick muscle cultures. In addition it was noted, in agreement with the observation of Coleman et al., (1978), that the use of NC 60/40 medium resulted in cultures which contained a high proportion of cross-striated myotubes whose appearance was superior to those obtained using alternative types of basal medium. In terms of the method of preparation of the cell suspension two factors were found to be important. As shown previously by Bullaro and Brookman (1976) cell suspensions prepared by enzymatic dissociation i.e. trypsinization, generally resulted in cultures whose quality with respect to development and maintenance were poorer than those prepared by mechanical dissociation i.e. use of a vortex. Bullaro and Brookman (1976) attributed part of this difference to differences in the viability of the cell suspensions. Initially

cell suspensions obtained by trypsinization were found to have higher viability (80-90%), but by 24 hours in culture the viability of these cells was less than 30%. In contrast suspensions obtained by mechanical dissociation although of initially lower viability (40-50%) did not deteriorate further during culture. Thus it appears that cells prepared by enzymatic dissociations may be irreversibly damaged and undergo slow death in culture. Interestingly, it has been suggested that the variability in the quality of different batches of horse serum already mentioned, may be related to their ability to inhibit the activity of the dissociating enzyme (Wallis et al., 1969). However, since variations in the quality of the serum are also noted when mechanically dissociated cells are used, serum components other than its inhibitory agent must also be involved. The other factor which was found to be important in obtaining good cell suspensions for culture was the use of preplating prior to inoculation of the dishes. This method is widely used (Hauschka, 1972) to enrich the cell suspensions for myoblasts by selectively removing non-myogenic cells, largely fibroblasts, which attach more rapidly than the myoblasts. Failure to use this step usually resulted in cultures of poorer quality, especially with respect to long term maintenance.

Identical procedure were used in the preparation of cultures from dystrophic embryos. In contrast to the results obtained by Askanas et al., (1971) and Allen and May (1979), we were unable to demonstrate any significant differences, with respect to the

morphology of growth and development, between normal and dystrophic cultures. However, these authors used breast muscle as the source of the tissue in contrast to the leg muscle used in the present study. Although no fibre type differentiation was noted in cultures of normal breast or leg muscle (Askanas, et al., 1972), the fact that the leg muscles tend to be less severely affected than the breast muscle cannot be discounted as the source of disagreement with Askanas et al., (1971). A more definitive study on the culture of leg and breast muscle from dystrophic embryos is required to resolve this point.

B. Myofibril Isolation Procedure

The basic problem to be overcome in devising a procedure for the isolation of myofibrils from muscle cultures is that as a consequence of the small amounts of starting material it is necessary to minimize losses in order to obtain sufficient material for analysis. The studies presented here indicate that the major source of losses which occurred using reported procedures (La Grange and Low, 1975; Bester and Gevers, 1976; Allen et al., 1978) were due to extensive shortening of the myofibrils which resulted in detachment from the culture dish. It was found that this problem could be overcome by use of a procedure to induce rigor requiring the use of the non-ionic detergent, triton-X 100. This reagent is widely used in protein chemistry and has previously been used in the isolation of myofibrils (Kohn, 1969; Zak et al., 1972; Etlinger and Fischman, 1973; Bester and Gevers, 1976; Allen et al., 1978). Striated muscle

fibers in culture when treated in this way became quite obviously rigid and retained a high degree of structural organization, as indicated by the distinct cross-striations. The mechanism of this "rigor" induction is unknown but it is most likely the result of selective solubilization of non-myofibrillar components containing lipid such as membranes by the triton-X100 buffer (Etlinger and Fischman, 1973; Etlinger, et al., 1976). The action of the detergent probably results in dissolution of the sarcolemma and subsequent solubilization of the intracellular membrane system and sarcoplasmic lipoproteins, leaving the structural elements relatively undisturbed. Presumably the failure of the detergent to solubilize the myofibrils is a consequence of the lack of phospholipids in the myofibril structure (Etlinger and Fischman, 1973). Included in these components which are released from the cytosol by the solubilization of retaining membrane will be ATP. Thus the loss of ATP or at least its considerable dilution by the detergent treatment could result in the induction of rigor as envisaged by Goll et al., (1970) in post mortem muscle. The observation that the presence of ATP (2 mM) in the RIB, results in rapid and extensive contraction of the myotubes would tend to support this suggestion. It is noteworthy that the rapid shortening in the presence of ATP was not prevented by the use of EDTA (to chelate Ca^{++}) in the RIB or even pretreatment with EDTA suggesting that the Ca^{++} mediated regulation of muscle contraction (Ebashi et al., 1969) was either too rapidly activated for the chelator to be effective or the regulatory system

was inactive in these cross-striated myotubes, (Hitchcock, 1970).

This latter suggestion may be related to the failure to observe significant quantities of TN-C and TN-I on the gels.

The fact that the tissue remained attached to the dish after induction of rigor (which would presumably have removed the sarcolemma) indicated that some detergent insoluble material was involved in anchorage of the cells. This was most probably collagen which has been shown to form an extensive extracellular network in mature muscle fibers (Borg and Caulfield, 1980). In addition this would account for the need to use the collagenase treatment as a method of detachment. One of the shortcomings of the procedure was the need to use this enzymatic method of detachment, since the presence of non-specific contaminating proteinases in the collagenase preparation could have given rise to proteolytic attack of the myofibrils during isolation. It is well known that many of the myofibrillar proteins particularly the Z-line are sensitive to trypsin (Harsanyi and Garamvolgyi, 1969) the principal contaminating activity (trypsin-like). It is therefore possible that a limited degree of proteolysis occurred during isolation and could account for the unusually low levels of α -actinin (Z-line) and absence of some of the regulatory protein. However the low level of these contaminants in the collagenase preparation and the short exposure time (5 minutes) would tend to argue against extensive proteolysis from this source.

The yield of myofibrils was also dependent on the mechanical force used to homogenize the detached material. Thus more vigorous

procedures resulted in a complete loss of myofibrils as judged by phase contrast examination, while gentler disruption gave the typical single sarcomere/short fragment mixture shown in Figure 17. This suggested that these culture myofibrils, unlike those obtained from adult muscle, may have been less structurally sound and therefore more susceptible to these treatments. Such lack of structural integrity could be due to the fact that these are relatively young myofibrils in the sense that they were only recently assembled within the cells from which they were derived. Consequently there may not have been sufficient time to consolidate the structure through establishment of all the necessary intramyofibrillar structural links to the extent they occur in more mature systems. The relative consistency of the myofibril composition after day 6 or 7 would suggest that all of the components are present but the results of such analysis cannot reveal any information about the nature of the forces involved in maintaining the structure. In addition the distinct structural differences in myofibrils from red and white muscle (Gauthier, 1970) suggest that neurotrophic influences, not present in the cultures, may have a role to play in consolidation of myofibrillar integrity. Alternatively it is possible that the observed fragility was a consequence of proteolysis during isolation eg. collagenase treatment, leading to a loosening of the structure. However, Kohn (1969) has used a similar purification step in isolating myofibrils from adult rat skeletal muscle and no such fragility was observed in these preparations. Whatever the explanation the

fact that the myofibrils appeared to fracture transversely into short fragments, may be related to the low levels of α -actinin observed on the gels.

With regard to the electrophoretic analysis of these preparations, they were on the whole similar to those obtained from adult muscle (Porzio and Pearson, 1977) but some differences were noted which deserve comment. Although the electrophoresis method used here was the same as that described by Porzio and Pearson (1977), we consistently observed better penetration of our samples, particularly the very high molecular weight material, into the gels. These intrinsic differences in the two systems may have been a result of differences in the purity of the reagents used. Thus although all of our reagents were electrophoresis grade, Porzio and Pearson (1977) repurified several of these materials before us. Therefore the presence of small amounts of impurities in our gels may have interfered with the cross-linking reaction to the extent that our gels were more porous. In this context it was noted that different batches of acrylamide resulted in slightly different mobilities of the proteins and this may have been responsible for the slight differences noted between the normal and dystrophic myofibrils. The lower levels of myofibrillar proteins obtained from dystrophic compared to normal cultures could be due to several factors including a smaller total myofibrillar pool of proteins and a lower efficiency of extraction in dystrophic cultures. With regard to these suggestions it was noted that the levels of extractable cell protein in

normal and dystrophic cultures between 7 and 10 days of age were similar but that dystrophic cultures appeared to contain slightly more fibroblasts suggesting that the muscle proteins would therefore constitute a smaller fraction of the protein in the cultures. In addition the presence of higher levels of contaminating fibroblasts could conceivably interfere with the isolation procedure and reduce the yields of myofibrils obtained. The apparent differences between the normal and dystrophic myofibrils with respect to actin and the regulatory proteins TM and TN-T was investigated by co-electrophoresis of normal and dystrophic samples (results not shown). The results indicated no differences in the mobility of actin but were inconclusive with respect to the regulatory protein. The reason for this was that the samples used in co-electrophoresis had been stored (-70°C) for some time before analysis with the results that the levels of regulatory proteins had diminished presumably as a result of proteolysis. Therefore further investigation will be required to establish whether there are real differences between the proteins or whether the observed differences are an artifact of electrophoretic analysis.

In terms of composition, the culture myofibrils differed in several respects from adult preparations analyzed by the same method. First the 305K component has not previously been reported and it was therefore considered that it may in fact have been a contaminant of the preparation. However since it formed a fairly consistent fraction of the material on the gels and the fact that it exhibited

a half-life identical to other myofibrillar proteins might suggest that it is an intrinsic component of the myofibril. In addition the virtually universal observation (Porzio and Pearson, 1977; Etlinger et al., 1976) of very high molecular weight material in adult myofibril preparations and the recent attempts to characterize this fraction (Wang et al., 1979) allow for the possibility that the 305K component is part of this fraction. The fact that the 305K component does not correspond directly to any of the proteins reported by Wang et al., (1979) could mean that the 305K component was derived by proteolysis which has been shown to occur in other myofibril isolation procedures (Etlinger et al., 1976). In this context the 140K component although of the same molecular weight as the C-protein (Porzio and Pearson, 1977) is present in rather higher quantities than would normally be expected. Thus this protein could be a fragment of a higher molecular weight protein (Porzio and Pearson, 1977; Etlinger et al., 1975), perhaps derived from the same source as the 305K component. In the attempts to determine at which stage of culture development the composition of the myofibril stabilized it was noted that the 305K component was absent in the very early post-fusion period. The reason for this developmental change is not known but it could be that this component is involved in the assembly of the myofibril. However further studies will be required to confirm or refute this suggestion.

C. Inhibitor Studies

Treatment of the cultures with the inhibitors was based on the procedures used by McGowan, et al., (1976). These worker had shown

that the inhibitors at concentrations of 50 ug/ml, were effective in delaying degeneration of normal and dystrophic chick muscle explant cultures, as judged by morphological and ultrastructural analysis. Adherence to this procedure necessitated the use of a higher molar concentration of leupeptin (104.2 uM). Pepstatin is of limited solubility in water (Umezawa et al., 1970), but this problem was overcome by titrating an aqueous suspension of the free acid with sodium hydroxide. This resulted in an almost instantaneous solubilization of the pepstatin suspension, undoubtedly due to the formation of the more soluble sodium salt (Umezawa et al., 1970). Stable stock solutions with concentrations as high as 8 mg/ml could be prepared in this way. Subsequently it was found that this procedure was essential for obtaining inhibition of protein degradation by pepstatin in the cultures. The failure of others in the past (Libby and Goldberg, 1978; Libby et al., 1979; Lockshin, 1975) to observe inhibition with pepstatin may well have been due to their use of different procedures to solubilize pepstatin.

In contrast to the protective action of these inhibitors on chick cultures reported by McGowan et al. (1976), the present study revealed either no observable differences or, in the case of dystrophic cultures treated with both inhibitors simultaneously, degeneration of the tissue. These differences cannot be accounted for in terms of differences in the types of culture systems used i.e. monolayer vs. explant, since the previous authors claimed that similar results were obtained using monolayer cultures. One possibility, at least in terms of treatments with a single inhibitor, is

that the effects observed by McGowan et al., (1976) occurred over a period of weeks, whereas in this study cultures were exposed to the inhibitors for a maximum of 72 hours. The degenerative effect produced by the combined use of the inhibitors on dystrophic cultures is an observation not reported previously. It was also noted that the sensitivity to this effect increased with the age of the cultures, a fact which accounted for our inability to obtain an acceptable preparation of myofibrils from 72 hour treated cultures. Since dystrophic cultures were not tested with single inhibitors at double their normal concentrations i.e. 100 ug/ml, it is not clear whether the degenerative effect was simply due to an increase in the total inhibitory capacity in the medium or whether there was a specific requirement for the presence of both inhibitors. In either event the combined use of the inhibitors was clearly toxic to the dystrophic muscle. As already mentioned, proteolysis, in general, appears to be a fundamental aspect of the cells' normal activities, and it could be that in this case the combined inhibitors blocked these fundamental processes to the extent that uninhibited proteolytic pathways became unusually active, or synthetic activities were also suppressed resulting in the observed degeneration. Perhaps the most interesting aspect of this phenomenon is that it was never seen in normal cultures. This would suggest that if the inhibitors are acting on the same systems in the normal cultures, then this tissue is not as critically dependent upon these systems for survival. However, since the normal embryos used to prepare cultures were not the precise genetic control for the dystrophic

strain it is possible that differences unrelated to the dystrophic mutation could account for the differences with respect to sensitivity to both inhibitors.

As a preliminary study, the effects of the inhibitors on the intracellular degradation of the TCA precipitable fraction of extractable cell protein were investigated. The isotope decay method was used because it provided a direct and relatively simple measurement of protein degradation in the cultures. There were however a number of problems, both technical and theoretical, associated with this approach. The principle of the isotope decay method is based on the idea that loss of radioactivity from the protein pool occurs as a result of degradation (Doyle and Tweto, 1975). Kinetic data obtained by this method is subject to error due to recycling of labelled precursor released during degradation and gives rise to apparently longer half-lives (Zak et al., 1979). This criticism is especially important when absolute values of degradation are required. Recycling can be overcome to some extent by increasing the concentration of unlabelled precursor in the system, as was done in this study. Thus through a process of dilution the chances of label being recycled are decreased. A possible disadvantage of the use of high extracellular methionine concentrations was that it could interfere with amino acid transport. Although this has been shown to occur in other systems (Christensen, 1964), the concentration required was greater than 2mM (the concentration used in the present study).

In addition other workers have used similar protocols in cultured muscle (Devlin and Emmerson, 1978; Whalen et al., 1976) without reporting any interference of amino acid transport. Furthermore in the experiments reported here, the controls were also subjected to these high levels of methionine.

With regard to the technical aspects of these experiments several points deserve comment. Estimating degradation through direct measurements of the protein fraction necessitated the use of destructive sampling techniques. Consequently only a single measurement could be carried out on each plate and therefore multiple determinations were required to establish individual points on the decay curves. Uncontrolled variations between plates were therefore potential sources of errors in these measurements. Some factors considered as likely sources of such variations were, differences in the size of the original inoculum, detachment of cells during culture, and variable losses during washing prior to extraction. However, the consistency of the protein yields/plate tend to suggest that such possibilities did not occur or that if they did they were fairly reproducible between individual plates. The choice of ^{35}S -methionine as a labelled precursor was based primarily on the facts that it had been shown by others (Devlin and Emmerson, 1978; Whelan et al., 1976) to be suitable for use in protein labelling experiments in muscle cultures and that it was relatively inexpensive. It was anticipated that since muscle proteins are

known to turnover slowly it may be necessary to follow loss of radioactivity for extended periods (days). This would require initially high levels of labelling in the cellular proteins which in turn would require the use of relatively large amounts of isotope. Initially high levels of labelling could also be achieved by labelling for a sufficiently long period (24 hours in this study). This would have the added advantage of ensuring labelling of the more slowly turning over proteins e.g. myofibrillar and avoid problems of selective labelling of more rapidly turning over proteins which might occur with shorter labelling periods. A problem associated with the use of ^{35}S -methionine as a label, in accordance with earlier studies of muscle protein metabolism in culture (Whalen et al., 1976; Emmerson, 1977; Devlin and Emmerson, 1978), was the possibility of contamination of the protein precipitates with methionine charged t-RNA. Brief treatment of the protein extracts with base, to release the methionine from the t-RNA, was therefore used in an attempt to avoid this difficulty. Finally the extraction procedure did not allow for discrimination between proteins of different cellular origins and the results obtained were therefore influenced to an unknown extent, by the presence of contaminating non-myogenic tissue in the cultures. The only estimates of contaminants which were obtained were from morphological examinations, which indicated that the cultures were not seriously contaminated but that the problem was increasingly obvious with the age of the

culture. As a consequence samples obtained in the latter stages of the experiment would have been more influenced by this factor.

The non-linearity of the control curves from normal cultures (Figure 12) is in contrast to the general observation that intracellular protein degradation is a first order process (Schimke, 1975; Schimke and Bradley, 1976). This non-linearity may reflect the complexity of the protein mixture in the precipitates and also the fact that the cultures were not in a steady state, as indicated by the protein determinations, (Table 6). The presence of DMSO did not alter the shape of the curve, suggesting that it had no effect on degradation in these studies. In agreement with results obtained by others using both rat and mouse skeletal muscle preparations (Libby and Goldberg, 1978; Kameyama and Etlinger, 1979), leupeptin was found to inhibit protein degradation in the cultures. The extent of inhibition, as measured in the cultures, was less than half of that obtained by Libby and Goldberg (1978) in their studies. Several factors, including differences in the muscle preparation and the assay systems used could account for this discrepancy.

An interesting observation, not made in the shorter term studies of Libby and Goldberg (1978) was that the effects of the inhibitors appeared to diminish with time. The effect can be seen most clearly by comparing the difference in the slopes, (expressed by $\Delta\text{cpm}/\text{hour}$ of the control and treatment curves) in the two intervals 0-24 hours and 24-72 hours (Table 16). In

TABLE 16

RATE OF DIVERGENCE OF CONTROL AND TREATMENT DECAY CURVES AS
ESTIMATED FROM SLOPES (Δ CPM/hour NORMAL)

	0-24 hrs	24-72 hrs
Leupeptin (L)	390	42
Pepstatin (P)	331	41
P + L	695	19

Values shown are the difference between control and
treatment values for slope (Δ CPM/hours) given in Table 4.

any given time interval the differences in the slopes will be an estimate of the differences in the rates of protein degradation under control and treatment conditions, which in turn will be a measure of the degree of inhibition. Thus, when the curves are parallel i.e. equal slopes, the difference is zero indicating there is no inhibition. As can be seen from Table 16 there was a marked decline in the degree of inhibition obtained with all three treatments. Two possible explanations of the phenomenon can be envisaged. First of all, the amount of inhibitor in the medium may have become depleted during the course of the experiment. However, as shown by the dose response curve the levels of inhibition obtained with half of the starting concentration i.e. 25 ug/ml actually resulted in slightly higher levels of inhibition. Thus, the amount of leupeptin remaining in the medium would have had to decrease by a considerable proportion (<50%) to account for the observed decrease in inhibition. If this is not the case, then the results would suggest that the cells had adapted or developed a resistance to the sustained presence of the inhibitors. Several general schemes concerning the nature of this resistance can be proposed. In the first instance the cells may have induced specific metabolic pathways as a means of detoxification, or the inhibitors' activity may have been neutralized extracellularly, perhaps through interaction with substances secreted into the medium. On the other hand, the putative resistance may have been more indirect in

that the cells, as a compensatory response activated leupeptin insensitive catabolic mechanisms or simply increased the activity of the inhibited system. With regard to this latter suggestion, Libby et al., (1979) reported increased levels of cathepsin B activity, (a likely site of action for leupeptin) in cultured fetal mice hearts, treated with leupeptin. The response was specific in that none of the other proteases assayed showed similar increases in the presence of leupeptin, and it was suggested that this might be a compensatory increase due to a sustained exposure to leupeptin. In their earlier studies of rat and mouse skeletal muscle, (Libby and Goldberg, 1978) no such increases in cathepsin B were noted. However, since the duration of these experiments was only a few hours, there may not have been sufficient time for this effect to become apparent. The decrease in inhibition with respect to time observed in the present study may well be a manifestation of similar phenomena in the cultured skeletal muscle. Some of the possible explanations proposed here could be tested by monitoring changes (decreases) in the inhibitory activity of the medium throughout the experiment and by determining whether fresh additions of leupeptin to previously treated cells could restore inhibition to the original levels. Constraints of time prohibited carrying out these experiments. In any event whatever the explanation it appears that longer term treatments with leupeptin do not result in a simple inhibition of protein degradation only, but probably

set in motion a complex sequence of events presumably destined to restore the cell to its normal metabolic equilibrium.

As estimated by the extractable cell protein method pepstatin was found to produce very similar levels of inhibition to that obtained with leupeptin (Table 4). This, as far as I am aware, is the first report of successful inhibition of protein degradation by pepstatin in a muscle system and is in contrast to the results obtained by others (Libby and Goldberg, 1978; Libby et al., 1979; Lockshin, 1975). The very close parallel between the decay curves obtained for pepstatin and leupeptin treatment suggests that both of these inhibitors elicited very similar responses from the cultured cells and, therefore, pepstatin would also be subject to the considerations already discussed for leupeptin. However, in view of their very different specificities with respect to inhibition of proteases (Umezawa, 1971; Barrett, 1974), it seems unlikely that they were producing these effects through action on precisely the same sites. This question of two distinct sites of action was also raised by the increase produced by the combined use of the inhibitors. The results of the dose response study indicated no significant changes in inhibition when the concentration of inhibitor was increased from 50-100 ug/ml. Thus, in the concentration range 50-100 ug/ml both inhibitors appeared to be producing close to the maximum response and the extra inhibition obtained by the combination would seem to indicate

inhibition at a second intracellular site.

The relatively broad specificity of leupeptin allows for several potential sites of action, of which the most commonly proposed is the lysosomal proteinase cathepsin B (Huisman et al., 1974b), known to be present in muscle (Bird, 1975). Several studies have examined the effects of leupeptin on the activity of this enzyme in a variety of in vitro cellular systems. Most of these studies demonstrated a decrease in cathepsin B activity coincident with a reduction in degradation upon treatment with leupeptin (Libby and Goldberg, 1978; Kameyama and Etlinger, 1979; Dean, 1979; Goldberg, et al., 1979). This led to the suggestion that cathepsin B, and presumably lysosomes, were components of the proteolytic systems in these tissues which may also be the case in the present study. However, as mentioned previously Libby et al., (1979) observed an increase in the activity of cathepsin B in leupeptin treated fetal heart cultures. The increase was concomitant with an inhibition of protein degradation, which would seem to suggest that cathepsin B (and lysosomes) is not involved in the leupeptin inhibited degradation in the fetal heart cultures. Another possible site of action of leupeptin in muscle is the Ca^{++} -activated neutral proteinase, which has been proposed as a candidate for a primary role in myofibrillar degradation (Dayton et al., 1975; Libby, 1977). No conclusions about the involvement of this particular enzyme in protein degradation in the cultures can be drawn from the

present study but it is of interest to note that Stracher et al., (1979) reported an inhibition of the total neutral proteinase activity (assayed in the absence of Ca^{++}) of chick muscle cultures treated with leupeptin. For the present, all that can be concluded from the work presented is that chick muscle cultures possess a leupeptin sensitive system which is involved in protein degradation. An important objective of future studies will be to define precisely the sites of action of leupeptin..

In evaluating possible sites of action for pepstatin two points are worthy of consideration. First, it is a highly specific inhibitor of carboxyl proteinases including cathepsins D and E, and it does not appear to exhibit activity against any other class of proteinases. Secondly, as shown by Knight and Barrett (1976) and Barrett (1974) pepstatin has no effect at pHs near neutral, requiring instead a fairly acid milieu. In view of these properties, by far the most likely site that can be proposed is the lysosomal acid proteinase, cathepsin D (Bird, 1975; Pennington, 1977). This enzyme appears to be the only known carboxyl proteinase in muscle, but the possibility that other, at present unknown proteinases of this class exist in muscle and could be targets for pepstatin, cannot be ruled out. Of particular interest in this context is the study by Stracher et al., (1979), which showed that 85% of the extractable acid proteinase activity of chick muscle cultures could be inhibited by pepstatin. However, as pointed out by Parsons et al., (1978),

who investigated lysosomal enzyme activities in rat muscle cultures, even small amounts of fibroblast contamination could account for considerable proportions of the total acid proteinase activity in the cultures since these cells are a much richer source of the activity, than muscle. Consequently, it is not clear whether the inhibited acid proteinase activity observed by Stracher et al., (1979) was truly of muscle origin. Nonetheless, until reasonable alternatives to cathepsin D can be demonstrated the most plausible explanation of the present results is that cathepsin D, and by association the lysosomal system, is involved in the observed protein degradation in chick muscle cultures. The inhibition of degradation by leupeptin and pepstatin could conceivably have led to a greater accumulation of protein in the treated cultures than that found in controls. However, failure to detect any such differences raised the possibility that inhibition of degradation may have resulted in a suppression of protein synthesis perhaps through some kind of feedback control mechanism (Waterlow et al., 1978). To test this possibility, the degree of incorporation of ^{35}S -methionine into the total cell protein in treatments and controls was measured and the values obtained were used as a comparative index of protein synthesis. The results indicated no significant differences in incorporation in leupeptin, pepstatin and leupeptin plus pepstatin treated cultures, suggesting that the inhibi-

tors had no significant effects on protein synthesis in the cultures. However, if protein synthesis in the treated cultures paralleled protein degradation, then we might predict on the basis of the observed decline in inhibition during the 24-72 hour interval a corresponding return of protein synthesis rates towards control levels during the same interval. Therefore, by carrying out the incorporation measurements after 36 hours of exposure to the inhibitors we may have chosen an interval during which the discrimination between treatments and controls was less than maximal. In this context it is interesting to note that the initial inhibitory effect on degeneration, as reflected in the amount of isotope retained in the protein pools, is not lost as inhibition declines with time.

Dystrophic cultures were similar to normal cultures in that they also exhibited a complex non-linear pattern of decay with respect to loss of radioactivity. However, they differed in a number of other respects. The reduced level of ^{35}S -methionine incorporation by dystrophic cultures, indicative of lower synthetic rates, is accounted for by the lower levels of protein in these cultures at the beginning of the experiment. Thus, the specific activities of the protein fractions from normal and dystrophic cultures were 36.4×10^3 cpm/mg and 37.5×10^3 cpm/mg respectively, indicating very similar specific rates of intracellular protein synthesis. However, in order to be certain that these values actually reflect similar rates of synthesis, it

would be necessary to correct for the specific activities of the respective intracellular precursor pools. As judged by the slopes (Δ cpm/hour) of the decay curves during the 0-24 hour interval the rates of degradation in the dystrophic cultures were lower than in normal controls. In addition, the levels of extractable protein in the dystrophic cultures at 0 and 24 hours were lower than in the corresponding normal controls. If, as suggested above, the rates of synthesis in normal and dystrophic cultures are similar, then the rate of accumulation should have been greater in dystrophic controls since rates of degradation in these cultures were lower than in normal controls. The actual values calculated from the data in Tables 6 and 7 are 2.67×10^{-2} mg protein/hour and 3.79×10^{-2} mg protein/hour for normal and dystrophic controls respectively during the first 24 h of the experimental period. One possible explanation for the difference in protein content could be that the viability of the original dystrophic cell suspension, was lower resulting in fewer cells attaching and subsequently developing. It might, therefore be of value to investigate the viability and plating efficiency of the dystrophic tissue.

The response of the dystrophic cultures to treatment with the inhibitors was similar to that obtained with normal cultures except that the degree of inhibition at 72 hours post labelling was less than in normal cultures and there was only a slight increase (at 24 hours) when the inhibitors were used in

combination. These lower levels of inhibition could perhaps be explained in terms of a more rapid activation of the postulated resistance mechanisms already discussed. Thus it is conceivable that leupeptin and pepstatin induced levels of inhibition equal to or even greater than that produced in normal cultures, but that the dystrophic cells responded more rapidly to re-establish the control rates of degradation. The more rapidly this occurs the smaller will be the difference in isotope content of the control and treatment protein fraction and the smaller the apparent degree of inhibition. The observation that the difference between the slopes of control and treatment curves obtained from dystrophic cultures (Table 17) was less than that observed for normal cultures would be consistent with a more rapid activation of the postulated resistance mechanism. In this context it is interesting to speculate that the cytotoxic effect produced by the combined use of the inhibitors in dystrophic cultures may have resulted from the more rapid, possibly excessive activation of the apparently more sensitive compensatory response. However this is purely conjecture and a more definitive study will be required to establish the mechanism of the observed cytotoxicity.

In summary then, the results of these studies on cell protein have shown that, leupeptin and pepstatin, probably through action on two different sites, were capable of inducing inhibition of protein degradation in both normal and dystrophic

TABLE 17

RATE OF DIVERGENCE OF CONTROL AND TREATMENT DECAY CURVES AS
ESTIMATED FROM SLOPES (Δ CPM/hour DYSTROPHIC)

	0-24 hrs	24-72 hrs
Leupeptin	201	5
Pepstatin	175	0
P & L	272	-7

Values shown are the differences between control and treatment values for slope (Δ CPM/hour) given in Table 5.

cultures, and that the effect diminished with time in the sustained presence of inhibitor. However, the presence of a small amount of contaminating non-muscle tissue in the cultures raised questions regarding the extent to which the observed effects were a true reflection of the behavior of the muscle and to what extent they reflected the presence of the contaminants. Therefore in order to examine more specifically the effects on the muscle, and to determine whether the major protein fraction of muscle, the myofibril, was involved in the observed responses, I undertook a study of myofibrillar degradation in the cultures. Degradation in these studies was estimated from the decay in specific activity of the labelled myofibrillar proteins under steady state conditions. The existence of a steady state was implied from the fact that the level of the extractable cell protein was constant and that the myofibrillar composition as judged by gel electrophoresis was also constant during the experimental interval. As a consequence of choosing this interval, (7th to 10th day in culture), the myofibrillar studies were carried out on cultures which were several days older than those used in total protein analysis.

Two measurements were required for the determination of specific activities. Radioisotope counting was carried out after extraction and solubilization of isotope into the cocktail. Several procedures for extraction and solubilization were tested and it was found that the use of peroxide treatments to digest

the gel slices containing the proteins, resulted in extremely high and stable backgrounds apparently due to chemiluminescence. I therefore, used the tissue solubilizer method of Paterson and Strohman (1972) which was free from problems of chemiluminescence and gave backgrounds of 50-100 cpm. Solubilization was carried out overnight to ensure maximum extraction of counts although it was found that this could be achieved fairly readily in two to three hours. A factor which may have assisted in the efficiency of extraction was the low cross-linking (100:1) of the acrylamide gels (Porzio and Pearson, 1977). This results in gels of higher porosity which would be amenable to good penetration of the tissue solubilizer. The second measurement necessary for specific activity determination, was an estimate of the protein content of each band to be analyzed. This was obtained from the areas under individual peaks of densitometric scans of the stained gels. The linearity of this densitometric response was not determined and it is, therefore, possible that this could give rise to errors in these estimates. However, examination of gels from different samples indicated that the overall loading and relative distribution of bands was fairly constant between samples suggesting that we were working in a fairly narrow range of protein concentrations for each band. In addition as already noted, the cultures were shown to be in a steady state with respect to total protein content during the experimental interval and since the myofibril isolation procedure was standardized the

yield of myofibrils was probably fairly consistent. Thus, it is unlikely that any large errors in protein determination resulted from the analysis of the densitometric scans.

In contrast to the results obtained in the analysis of the total cell protein, the decay curves from normal controls were found to be linear when plotted semilogarithmically, suggesting first order decay kinetics. This difference can most probably be explained in terms of the fact that the system was in a steady state and that single, pure proteins as opposed to a complex mixture were being analyzed. The first order kinetics are inconsistent with the suggestion that the myofibril as a whole exhibits a definite life-span and turns over as a unit (Dreyfus et al., 1960). If such were the case, then we would have expected to see retention of a constant level of label followed by a rapid drop after an interval equivalent to the life-span of the organelle, instead of the exponential decline observed. On the other hand, the results are compatible with the proposal that the individual components of the myofibril turn over independently (Low and Goldberg, 1973; Zak et al., 1977). However, the similarity in turnover rates of MHC, 305K and 140K could be interpreted as meaning that these proteins, perhaps through location in a particular component of the myofibril, are themselves turned over as a unit. However, as pointed out previously, it is possible that the 140K component is a fragment of the MHC, in which case only two out of three

distinct molecular species examined exhibit similar half-lives. It would, therefore, be rather premature to conclude on this basis that turnover occurs as a unit. On the contrary, the fact that half-lives of actin ($t_{1/2} = 50.3$ hrs) and MHC ($t_{1/2} = 38.4$ hrs), quantitatively the two most important proteins of the myofibril, are quite different, would suggest that the major proteins of the myofibril turn over independently in the cultured muscle. It is also interesting to note that actin with a molecular weight of 48,000 daltons exhibits a longer half-life than the high molecular weight components, in keeping with the general observation that high molecular weight proteins turn over more rapidly than low molecular weight proteins (Schimke, 1975; Schimke and Bradley, 1976; Goldberg and St. John, 1976). Thus, the data obtained here is consistent with a model in which the components of the contractile apparatus are synthesized and degraded independently.

With respect to the half-lives, the values obtained are somewhat shorter than those reported for muscle proteins in non-culture (Zak et al., 1977; Martin et al., 1977; Wikman-Coffelt et al., 1973; Low and Goldberg, 1973) and culture systems (LaGrange and Low, 1976; Rubinstein et al., 1976; Walker and Strohman, 1978). The reasons for these differences are not clear but several possible explanations can be proposed. In the first instance, the more rapid turnover rates may represent a response of the muscle to the specific in vitro conditions used in this study. Thus, cells maintained in this essentially adverse

environment may be required to turn over their constituent proteins more rapidly in order to combat the effects of this situation. However, others (LaGrange and Low 1976; Rubinstein et al., 1976; Walker and Strohman, 1978) using similar culture systems have reported half-lives of 5-6 days for actin and myosin. In the study by Walker and Strohman (1978) on MHC turnover in chick muscle cultures, it was noted that the presence of cytosine arabinoside resulted in an increase in the turnover rate of MHC and a half-life of 36 hours similar to that obtained in these studies, was reported. Since cytosine arabinoside was also used in these studies, it is possible that the shorter half-lives obtained are a reflection of the use of this agent. The nature of the mechanism involved in this cytosine arabinoside induced increase in turnover is unknown. On the one hand, it could be due to a direct effect of the drug on muscle as has been observed by others (Thomson and Dryden, personal communication). Alternatively, the influence on turnover could be an indirect effect resulting from the elimination of dividing cells which through some unspecified cell/cell interaction with muscle could have influenced protein turnover. In either event, caution must be exercised in drawing conclusions about the relationship of turnover under these conditions and the process as it occurs in vivo. Another factor which may have contributed to the shorter half-lives was the use of high concentrations of unlabelled

methionine in the post-labelling medium to reduce the risk of recycling, which could give rise to longer half-lives. Since both cytosine arabinoside and increased concentrations of cold leucine were used in the study by Rubinstein et al., (1976) then none of the above suggestions can account for the differences in half-lives observed. One possible explanation is that the high ionic strength extraction procedure used by these workers to isolate the contractile proteins would have resulted in extraction of contractile proteins from non-myofibrillar as well as myofibrillar sources (Adelstein et al., 1972; Ip and Fischman, 1978). Since the subsequent analysis of this fraction on SDS-PAGE would most probably have failed to resolve the contaminants from the myofibrillar proteins this would have influenced the half-life values obtained. If the contaminating protein turned over more slowly then the apparent half-lives of the myofibrillar fraction would be shorter. In contrast, the myofibril isolation procedure used in the present study was not subject to contamination of this sort and is in this respect a better analysis of the myofibril turnover. In addition to this difference between the studies, the double isotope method of Arais (1969) was used by Rubinstein et al., (1976) to determine the half-lives of the proteins. As pointed out by Zak et al., (1977) it is necessary to optimize the labelling conditions in this procedure to avoid errors leading to apparently longer half-lives, and it is not clear from the data presented whether the

conditions of the study met this criterion. The same arguments may be proposed to account for the differences between this study and that of LaGrange and Low (1976) who also used the double isotope approach.

Myofibril turnover in dystrophic cultures was similar to that in normal cultures. This is in contrast to the situation in vivo and in other in vitro models of muscles, where a more rapid turnover of the myofibril of dystrophic animals has been observed (Goldberg et al., 1977, Weinstock et al., 1969, Ionasecu et al., 1971 and Rourke 1975). In particular, the work of Rourke (1975) demonstrated increased rates of both synthesis and degradation of MHC in muscles of 15-23 day post hatched dystrophic chickens compared to that in normal controls. A number of facts may account for the difference between these results and those obtained in the present study. Thus, Rourke's study was carried out in vivo on the breast muscle of post hatched birds, while the present studies were carried out in vitro on tissue originating from the leg muscle of embryos. In addition, it is worth observing that even the increased rates of turnover observed by others (Rourke 1975, Goldberg et al., 1977, Weinstock et al., 1969, Ionasescu et al., 1971) in dystrophic muscles is slower than the rates observed in both the normal and dystrophic cultures in the present work. Thus, it can be suggested that higher rates of turnover may be a consequence of studying the muscle in a culture situation. Muscle in vivo, whether normal

or diseased, will be subject to control through a variety of homeostatic mechanisms and under such conditions diseased and normal muscle obviously exhibit differences particularly with respect to protein turnover. The removal of some of these controls by incubation in vitro could conceivably lead to changes in the overall metabolic profile of the cell. These changes may predominate to the extent that differences observable in vivo are masked in the culture environment. In this context, it is interesting to note that Goldspink (1977) observed a net positive nitrogen balance, attributed to increased protein synthesis, in incubated isolated dystrophic hamster diaphragms which in vivo had exhibited a very obvious net negative nitrogen balance as judged by loss of weight and overall protein content. The reason for this changeover from negative (in vivo) to positive (in vitro) nitrogen balance in this study could only be ascribed to the effects of the conditions in vitro. An analagous situation could occur in the muscle cultures. Another factor which may account for my failure to see any differences between normal and dystrophic myofibrillar turnover is that leg instead of breast muscle was used as the source. In adult birds affected by the disease, the breast is more seriously affected than the leg (Monckton, 1978). Thus, leg muscle may not express dystrophy in culture sufficiently to allow for distinction between it and normal muscle. However, as pointed out, Askanas et al., (1972) and Rubinstein and Holtzer (1979) were unable to

obtain evidence for differentiation with respect to fibre type on aneural cultures of chick leg and breast muscle, which may be necessary for the full expression of the diseased state.

Myofibrils from normal controls and inhibitor treatments exhibited no obvious differences with respect to banding pattern and relative distribution of components, as judged by electrophoresis. This finding was somewhat disappointing since it had been speculated that treatment with the inhibitors could have resulted in the accumulation of intermediates in the myofibril disassembly process allowing some speculation on the nature of this mechanism. Several factors could have been responsible for the failure to observe any difference. First, if such intermediates do occur and it is by no means certain that they do, then it is possible that they separate very rapidly from the myofibril and would not, therefore, appear in our preparation. On the other hand, the accumulation of proteolytic fragments which did not dissociate from the myofibrils has been observed in studies of post-mortem muscle (Hay et al., 1973). It is also possible that the specificity of the inhibitors was not conducive to the production of the type of inhibition required for the accumulation of such intermediates. The fact that leupeptin is an inhibitor of the Ca^{++} activated neutral protease (Libby, 1977) which has been proposed as a probable initiator in myofibril disassembly (Dayton et al., 1975) might suggest that the formation of such intermediates was actually blocked,

thus, excluding the possibility of their accumulation and subsequent detection. In this context, Etlinger et al., (1979) have demonstrated that leupeptin, possibly through its action on the Ca^{++} activated enzyme, reduced the amount of easily releasable filaments thought to be disassembly intermediates, in preparations of myofibrils from treated rat skeletal muscles. Although I cannot rule out the involvement of a pepstatin sensitive acid proteinase in the initiation of myofibril disassembly (Bird et al., 1978), the prospects of such an eventuality seem unlikely. Thus, the accumulation of the postulated intermediate would have been more probable with this inhibitor. Perhaps then the plausible explanation for these negative results is that such intermediates do not exist. As pointed out in the introduction, there is no evidence to suggest that peptide bonds are involved in the intermolecular forces which maintain myofibrillar integrity and as such there may be no need to invoke the action of proteinases in the disassembly process. This would imply that the inhibition of myofibrillar turnover observed in the presence of both inhibitors was the result of their action at a post-disassembly stage.

The results of parallel studies on dystrophic cultures were similar with the exception that myofibrils from cultures treated with both inhibitors behaved quite differently on electrophoresis. In this case, the gels although exhibiting some resolution of the usual banding pattern, were diffusely stained throughout.

Such staining indicated the presence of protein throughout the gel rather than resolved into distinct bands. The most likely explanation of this phenomenon is that proteolysis resulting in peptide fragments with a spectrum of molecular weights corresponding to the range covered by the diffusely stained region of the gel were being produced and interestingly were sedimenting with the myofibril preparation. This extensive proteolysis appeared to be a reflection of the cytotoxic state of these cells. It is questionable whether the proteolytic mechanisms at work in this situation are akin to those involved in the turnover process and, therefore, the proteolytic fragments responsible for the diffuse staining are most probably not intermediates of turnover.

All of the inhibitor treatments tested, resulted in a decrease in the rate of decay of specific activity with respect to controls. In view of the results obtained on the total cell protein analysis, the most likely explanation for these results is that the inhibitors blocked protein degradation, although the possibility that inhibition of synthesis contributed to this effect, cannot be ruled out. Thus, with no change in degradation and a complete inhibition of synthesis the specific radioactivity of cell protein, measured as TCA precipitable material, remains constant while the size of the particular protein pool decreases. On the other hand, a complete shutdown of degradation without

alteration in synthesis would still lead to a decay in the specific radioactivity of cell protein but at a lower rate than the steady state control since in this case there is no loss of isotope from the pool simultaneous with dilution through synthesis. As a consequence, under these conditions there will be an increase in the size of the pool. The increase in the amount of material on gels of standard myofibril preparations from inhibitor treated normal cultures may be a reflection of an increased myofibrillar pool induced through inhibition of degradation and leading to higher yields of myofibrils for analysis by electrophoresis. The fact that no such increases were induced by the inhibitors in dystrophic cultures is interesting. One possible explanation for this difference could be that as suggested earlier, the postulated resistance mechanism is more rapidly activated in dystrophic cultures, thus, preventing any significant increases in the myofibrillar pool in dystrophic cultures.

Compared to the results obtained from total cell protein analysis the extent of inhibition for the myofibrillar proteins is considerably higher. Several reasons could account for this difference. First of all, the proteolytic mechanisms involved in myofibril degradation may be particularly susceptible to these inhibitors leading to greater degree of inhibition than that observed for other proteins in the tissue. Secondly, as already pointed out the effect may not simply be due to inhibi-

bition of degradation but could also result from inhibition of synthesis either as the result of a direct effect of the inhibitors, or through some other indirect mechanisms e.g. feedback control linked through degradation. The results of total cell protein analysis suggested that this may, in fact, occur and if the extent to which this affected myofibrillar protein was greater than that for other cellular protein, then this could account for the greater inhibition observed with these proteins. The relative contributions of inhibition of synthesis and degradation cannot, however, be determined from the present results. A third factor which may account for the rather high levels of inhibition is that the increased amounts of protein on gels from inhibition treatments could have resulted in underestimates of protein concentrations from the densitometric tracings of the response of the densitometer was in a plateau region at these concentrations. These underestimates would have resulted in erroneously high specific activities and, thus, overestimated the extent of inhibition. However, since the increased pool size leading to these errors would be consequent upon inhibition then the errors would only influence the degree of inhibition observed but would not alter the fact that inhibition had, in fact, been induced. In this context it is also worth noting that the high levels of inhibition were observed in dystrophic cultures which did not exhibit any increases in the levels of myofibrillar proteins. Thus in this case the level of inhibition

cannot be accounted for in terms of errors in protein quantitation. Another possibility is that the inhibitors increased the extent of recycling of labelled precursor. In the case of myofibrils labelled precursors could mean not only the ^{35}S -methionine used in the original labelling, but also intact labelled monomers obtained from disassembly. If the presence of the inhibitors resulted in an increased reutilization of labelled macromolecules or monomers from disassembly then the rate of decline of specific activity would be reduced. However, until the existence of such macromolecular pools can be demonstrated (Zak et al., 1976) and the extent to which recycling occurs in this system, then the possible contribution of this phenomenon to the present results cannot be determined.

With regard to possible sites of action, the same considerations as were discussed for the total cell protein analysis, apply. Thus, for leupeptin the most likely targets are cathepsin B and the Ca^{++} activated neutral proteinase while for pepstatin cathepsin D is the most probably susceptible enzyme. This being the case, the results are consistent with a lysosomal involvement in the turnover of myofibrillar proteins in the cultures as has been suggested by Bird et al., (1978) for muscle in vivo, but whether this involvement is at the level of myofibrillar disassembly or at some other stage cannot be determined from the present data. However, in view of the relatively non-specific

nature of the lysosomal system (Bird, 1975) it seems that these enzymes are more suited to the terminal stages of the degradative process i.e. hydrolysis of peptides leading to amino acid release (Huisman et al., 1974b).

The diminishing effectiveness of the inhibitors during the experimental interval which was also noted in the total cell protein studies suggests that similar mechanisms may have been involved here. Other parallels between the two sets of investigation were noted. Thus, myofibrillar proteins from dystrophic myofibrils appeared to be less affected by the inhibitor at 72 hours and the degree to which control levels had been restored in the 24-72 hour interval was greater than for normal myofibrils. As discussed, the relevance of these differences to dystrophy is not known but it seems possible that it could be a reflection of a pathological process which is retained and detectable in the culture situation.

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